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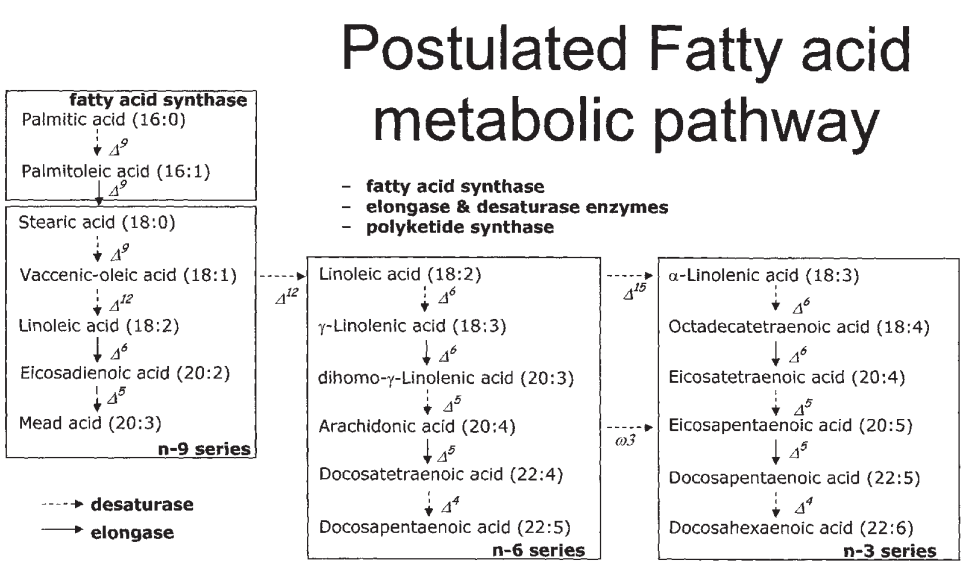
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(54) **Title:** OIL PRODUCING MICROBES AND METHODS OF MODIFICATION THEREOF



(57) **Abstract:** Disclosed are compositions and methods related to oil producing microbes that can produce unsaturated fatty acids which can be purified and used. Also disclosed are methods to modify said fatty acid concentrations within total lipid compositions using fatty acid production antagonists which either inhibit or stimulate certain components of the classical fatty acid production pathway thought to exist within oil producing microbes.

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OIL PRODUCING MICROBES AND METHODS OF MODIFICATION THEREOF

1. This application claims the benefit of and priority to U.S. Provisional Application No. 60/821, 084, filed August 1, 2006 which is incorporated by reference herein its entirety.

I. BACKGROUND

2. There is overwhelming scientific evidence that (n-3) highly unsaturated fatty acids such as docosahexaenoic acid (DHA) have a positive effect on cardio-circulatory diseases, chronic inflammations and brain disorders, while eicosapentaenoic acid, also an n-3 fatty acid, has been noted to perform functions as an intermediate metabolite within the eicosanoid steroids, such as prostaglandins, leucotrienes or the like.

3. Currently, the main source of these highly unsaturated fatty acids is fish, with EPA and DHA noted within various blue fish (such as sardines and tuna) at amounts around 20% and 10%, respectively. It is believed that such a fatty acid profile occurs through the natural selection of optimal ratios for optimal performance within each species of fish. Yet, if one intends to use fish oil as the sole source of these lipids, several disadvantages exist, such as problems with flavor taint, uncontrollable fluctuations in availability and natural fish oil content variability. In addition, if one intends to obtain a highly purified (n-3) or (n-6) oil from these sources, it is very difficult to preferentially separate and purify.

II. SUMMARY

4. Disclosed are compositions and methods related to a eukaryote of the order Thraustochytriales and family Thraustochytriaceae which when cultured produce quantities of unsaturated fatty acids, such as omega 3 (n-3) and/or omega 6 (n-6) oils, such as DHA, EPA and DPA, capable of being purified and used as all such compositions are used and more, because of their means of production.

5. Disclosed are compositions and methods related oil producing microbes, which when cultured produce quantities of unsaturated fatty acids, such as omega 3 (n-3) and/or omega 6 (n-6) oils, such as DHA, EPA and DPA, capable of being purified and used as all such compositions are used and more, because of their means of production

6. Also disclosed are methods to modify said fatty acid concentrations within total lipid compositions using fatty acid production antagonists which either inhibit or

stimulate certain components of the classical fatty acid production pathway thought to exist within oil producing microbes.

III. BRIEF DESCRIPTION OF THE DRAWINGS

7. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments and together with the description illustrate the disclosed compositions and methods.

8. Figure 1 shows the proposed metabolic pathway for the production of polyunsaturated fatty acids (PUFAs) for the disclosed oil producing microbes.

9. Figure 2 shows the fatty acid profile of ONC-T18 grown in medium containing 2 g L^{-1} yeast extract, 8 g L^{-1} L-glutamate, 6 g L^{-1} sea salt and 60 g L^{-1} glucose in 3 different types of fermentation: agar plate (1.5% agar, 25°C, 27 days), flasks (50 ml in 250 ml flask, 120 RPM, 25°C, 3 days) and 5 L bioreactor (4 lpm air, pO_2 90%, 25°C, 3 days).

10. Figure 3 shows typical biomass, total fatty acid (TFA), DHA production and glucose utilization of *Thraustochytrium* sp. ONC-T18 maintained in a 5 L bioreactor for 168 h with medium composed of 60 g L^{-1} glucose, 2 g L^{-1} yeast extract, 8 g L^{-1} glutamic acid and 6 g L^{-1} salt (4 lpm air, pO_2 90%, 25°C, pH 7-9).

11. Figure 4 shows the evolutionary relatedness of *Thraustochytrium* sp. ONC-T18 based on the 18S rRNA gene to several Thraustochytrids of Japanese origin (*Thraustochytrium* CHN-1, Thraustochytriidae MBIC 11093 and Thraustochytriidae N1-27) and a *T. striatum* strain T91-6.

12. Figure 5 shows ONC-T18 was grown on various Nitrogen sources, e.g. yeast extract with various grades, L-glutamate, corn steep liquor, etc. L-glutamate alone did not promote biomass, total fatty acid (TFA) or DHA production. However, in the presence of L-glutamate, various N sources, including yeast extract and corn steep liquor, gave better results than without L-glutamate. (Related to example 11 Table 7).

13. Figure 6 shows ONC-T18 was grown on various Carbon sources, and monosaccharides such as glucose etc., sucrose and molasses gave high biomass, TFA and DHA. (Related to Table 6 & 7).

14. Figure 7 shows water soluble antioxidant, phytohormones or sodium acetate (simple carbon) did not improve biomass, TFA or DHA results compare to standard medium alone.

15. Figure 8 shows the difference between the fermentation of ONC-T18 at 18°C and 25°C. The lower temperature (i.e. 18°C) resulted in a significantly higher glucose uptake, half the TFA and comparable biomass and DHA levels to that of the control culture. Thus, fermentation at 25°C was found to be more efficient than at 18°C.

16. Figure 9 shows ONC-T18 was fed with pure oleic acid. The fatty acid profile of ONC-T18 (fed with pure oleic acid) showed that most of the oleic acid was converted into DHA, with an elevated level of EPA compare to glucose-fed ONC-T18.

17. Figure 10 shows the effects of toluic acid on the fatty acid composition of ONC-T18. The fatty acid profile of ONC-T18 (fed with 0.2 g/L Toluic acid) showed that the compound was able to increase the proportion of EPA within the profile while decreasing the proportion of DPA (n6). Values are the mean of four experiments comparing 0.2 g/L toluic acid to a control.

18. Figure 11 shows the gas chromatography results of culturing ONC-T18 with examples of fatty acid production antagonists.

19. Figure 12 shows the effect of the addition of Norflurazon to ONC-T18 fatty acid profile. Figure 12 shows that the proportion of PUFA increased as the amount of Norflurazon added was increased.

20. Figure 13 shows that when ONC-T18 was exposed to Norflurazon, the carotenoid content decreased, paralell with a DHA increase.

21. Figure 14 shows that when oleic acid was added into the medium of ONC-T18, more DHA content was observed, especially when associated with growth (indicated by the presence of high N and low N).

22. Figure 15 shows that when cerulenin was added into the medium of ONC-T18, while a change in the % of each fatty acid to total fatty acid content was not observed, when total fatty acid concentrations as relates to biomass (mg/g) was taken into account increases in both C16:0 and DHA were noted.

IV. DETAILED DESCRIPTION

23. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, and as such may, of

course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

24. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the method and compositions described herein. Such equivalents are intended to be encompassed by the claims below.

A. Definitions

25. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

26. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

27. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed then "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point "15" are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is

also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

28. By "reduce" or other forms of reduce means lowering of an event or characteristic. It is understood that this is typically in relation to some standard or expected value, in other words it is relative, but that it is not always necessary for the standard or relative value to be referred to. For example, "reduces fatty acid concentrations within total lipid compositions" means lowering the amount of fatty acid concentrations within total lipid compositions within an oil producing microbe relative to a standard or a control. It is understood that unless specifically indicated otherwise, a compound or composition or condition can be reduced relative to another compound or composition or condition.

29. By "increase" or other forms of increase means increasing of an event or characteristic. It is understood that this is typically in relation to some standard or expected value, in other words it is relative, but that it is not always necessary for the standard or relative value to be referred to. For example, "increase fatty acid concentrations within total lipid compositions" means increasing the amount of fatty acid concentrations within total lipid compositions within an oil producing microbe relative to a standard or a control. It is understood that unless specifically indicated otherwise, a compound or composition or condition can be increased relative to another compound or composition or condition.

30. By "inhibit" or other forms of inhibit means to hinder or restrain a particular characteristic. It is understood that this is typically in relation to some standard or expected value, in other words it is relative, but that it is not always necessary for the standard or relative value to be referred to. For example, "inhibits a desaturase" means hindering, restraining or causing a ceasation of activity in the amount of desaturase activity of the enzyme that takes place relative to a standard or a control. It is understood that unless specifically indicated otherwise, a compound or composition or condition can be inhibited relative to another compound or composition or condition.

31. By "prevent" or other forms of prevent means to stop a particular characteristic or condition. Prevent does not require comparison to a control as it is typically more absolute than, for example, reduce or inhibit. As used herein, something could be reduced but not inhibited or prevented, but something that is reduced could also be inhibited or prevented. It is understood that where reduce,

inhibit or prevent are used, unless specifically indicated otherwise, the use of the other two words is also expressly disclosed. Thus, if inhibits the desaturase activity of a desaturase is disclosed, then reduces and prevents desaturase activity of a desaturase are also disclosed.

32. The term "therapeutically effective" means that the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

33. The term "carrier" means a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

34. Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

35. The term "cell" as used herein also refers to individual microbial cells, individual oil producing microbe cells, or cultures derived from such cells. A "culture" refers to a composition comprising isolated cells of the same or a different type.

36. The term "metabolite" refers to active derivatives produced upon introduction of a compound into a biological milieu, such as a patient.

37. When used with respect to pharmaceutical and nutraceutical compositions, the term "stable" is generally understood in the art as meaning less than a certain amount, usually 10%, loss of the active ingredient under specified storage conditions for a stated period of time. The time required for a composition to be considered stable is relative to the use of each product and is dictated by the commercial practicalities of producing the product, holding it for quality control and inspection, shipping it to a wholesaler or direct to a customer where it is held again in storage before its eventual use. Including a safety factor of a few months time, the minimum product life for pharmaceuticals is usually one year, and preferably more than 18 months. As used herein, the term "stable" references these market realities and the

ability to store and transport the product at readily attainable environmental conditions such as refrigerated conditions, 2°C to 8°C.

38. References in the specification and concluding claims to parts by weight, of a particular element or component in a composition or article, denotes the weight relationship between the element or component and any other elements or components in the composition or article for which a part by weight is expressed. Thus, in a compound containing 2 parts by weight of component X and 5 parts by weight component Y, X and Y are present at a weight ratio of 2:5, and are present in such ratio regardless of whether additional components are contained in the compound.

39. A weight percent of a component, unless specifically stated to the contrary, is based on the total weight of the formulation or composition in which the component is included.

40. "Isolating" and any form such as "isolate" refer to a situation where something is in a form wherein it can be manipulated or further purified. Isolated and its forms indicates that something is in a current state which is different than a previous state. For example, a ribosomal RNA molecule can be "isolated" if it is, for example removed from an organism, synthesized or recombinantly produced. Often, the "isolation" of one thing is in relation to something else. For example, an oil producing microbe as discussed herein can be isolated as discussed herein, by, for example, culturing the oil producing microbe, such that the oil producing microbe survives in the absence of appreciable amounts (detectable) of other organisms. It is understood that unless specifically indicated otherwise, any of the disclosed compositions can be isolated as disclosed herein. In addition, the lipid produced from the oil producing microbes as discussed herein, can be isloted from all or part of the oil producing microbe.

41. "Purify" and any form such as "purifying" refers to the state in which a substance or compound or composition is in a state of greater homogeneity than it was before. It is understood that as disclosed herein, something can be, unless otherwise indicated, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% pure. For example, if a given composition A was 90% pure, this would mean that 90% of the composition was A, and that 10%

of the composition was one or more things, such as molecules, compounds, or other substances. For example, if a disclosed eukaryotic microorganism, for example, produces 35% DHA, this could be further "purified" such that the final lipid composition was greater than 90% DHA. Unless otherwise indicated, purity will be determined by the relative "weights" of the components within the composition. It is understtod that unless specifically indicated otherwise, any of the disclosed compositions can be purified as disclosed herein.

42. "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

43. "Primers" are a subset of probes which are capable of supporting some type of enzymatic manipulation and which can hybridize with a target nucleic acid such that the enzymatic manipulation can occur. A primer can be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art which do not interfere with the enzymatic manipulation.

44. The term "omega-3", "n-3", "ω-3", "omega n-3", and "n-3 series" refers to a family of polyunsaturated fatty acids which have in common a carbon-carbon double bond in the ω-3 position. For example, the term "omega-3" signifies that the first double bond exists as the third carbon-carbon bond from the terminal methyl end (ω) of the carbon chain.

45. The term "omega-6, "n-6", "ω-6", "omega n-6", and "n-6 series" refers to a family of polyunsaturated fatty acids which have in common a carbon-carbon double bond in the ω-6 position. For example, omega-6 signifies that the first double bond exists as the sixth carbon-carbon bond from the terminal methyl end (ω) of the carbon chain.

46. The term "omega-9, "n-9", "ω-9", "omega n-9", and "n-9 series" refers to a family of polyunsaturated fatty acids which have in common a carbon-carbon double bond in the ω-9 position. For example, omega-9 signifies that the first double bond exists as the ninth carbon-carbon bond from the terminal methyl end (ω) of the carbon chain. All double bonds are in the cis-configuration, i.e. the two hydrogen atoms are on the same side of the double bond.

47. The term "oil producing microbe" refers to a microbe capable of producing oil as part of its biomass. An "oil producing microbe" can be a eukaryotic or prokaryotic. For example, an oil producing microbe can be an oleaginous organism,

that is one which are able to accumulate oils and/or fats above 20% of their dry biomass. An oil producing microbe can be a diatom, a microalgae, a fungi, a bacterium, a protist, a yeast, a plant (both terrestrial and marine) or an algae.

48. The term "oleaginous organism" refers to any organism that can prodcue more than 20% of its biomass as lipids. For example, an "oleaginous organism" can be a diatom, a microalgae, a fungi, a bacterium, a protist, a yeast, a plant (both terrestrial and marine) or an algae.

49. The term "ketocarotenoid" refers to a keto group-containing carotenoid.

50. The term "isoprenoid" or "terpenoid" refers to the compounds or any molecule derived from the isoprenoid pathway including 10 carbon terpenoids and their derivatives, such as carotenoids and xanthophylls.

51. The term "carotenoid" refers to a compound composed of a polyene backbone which is condensed from five-carbon isoprene unit. Carotenoids can be acyclic or terminated with one (monocyclic) or two (bicyclic) cyclic end groups. The term "carotenoid" may include both carotenes and xanthophylls. A "carotene" refers to a hydrocarbon carotenoid. Carotene derivatives that contain one or more oxygen atoms, in the form of hydroxy-, methoxy-, oxo-, epoxy-, carboxy-, or aldehydic functional groups, or within glycosides, glycoside esters, or sulfates, are collectively known as "xanthophylls". Carotenoids that are particularly suitable in the present invention are monocyclic and bicyclic carotenoids.

52. The term "keto group" or "ketone group" will be used interchangeably and refers to a group in which a carbonyl group is bonded to two carbon atoms: R_2CO (neither R may be H).

53. The term "fatty acid production antagonists" as used herein refers to fatty acid subunits and fatty acid pathway modulators.

54. The term "fatty acid subunit" or "fatty acid subunits" as used herein refers to the 16, 18, 20, 22 or 24 carbon fatty acids involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. For example, "fatty acid subunits" can include any of the 16, 18, 20, 22 or 24 carbon fatty acids as shown in Figure 1.

55. A "fatty acid subunit" can also refer to any 16, 18, 20, 22 or 24 carbon fatty acids having 1, 2, 3, 4, 5 or 6 unsaturations involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. Further embodiments

can include odd numbered carbon fatty acids, with or without unsaturations such as 15, 17, 19 and 21 carbon fatty acids found within marine organisms.

56. The term "fatty acid pathway modulators" as used herein refers to compounds capable of inhibiting one or more of the enzymes involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes.

57. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

58. Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular species of the family Thraustochytriaceae is disclosed and discussed and a number of modifications that can be made to a number of organisms including species of the family Thraustochytriaceae are discussed, specifically contemplated is each and every combination and permutation of these species from the family Thraustochytriaceae and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional

steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

B. Compositions

59. Disclosed are oil producing microbes of the order Thraustochytriales, preferably *Thraustochytrium* or *Schizochytrium* species, having the ability to produce lipids, such as fatty acids, such as unsaturated fatty acids, such as omega-3 fatty acids, such as omega-6 fatty acids, and omega-9 fatty acids, such as the (n-3) series of docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and eicosapentaenoic acid (EPA), (n-6) series of DPA and the (n-9) series of palmitic and stearic acids.

60. The disclosed oil producing microbes can also produce antioxidants, such as but not limited to the carotenoid compound carotene, (for example β-carotene) and the xanthophylls compounds, astaxanthin, zeaxanthin, beta-cryptoxanthin, canthaxanin, and echinenone.

61. Also disclosed are conditions for the isolation and growth of the oil producing microbes. For example, herewith are heterotrophic growth conditions for production of the disclosed lipids and antioxidants, for example, both individually and cumulatively. Accordingly, it is possible through the use of the disclosed oil producing microbes, to effectively produce the (n-3) series of DHA, EPA and DPA and/or the (n-6) series of DPA and/or the carotenoid series of antioxidant compounds and/or the xanthophylls series of antioxidant compounds, which are useful as or within nutraceuticals, food additives, pharmaceuticals or industry.

62. Disclosed are compositions comprising an oil producing microbe comprising or consisting of a *Thraustochytrium* species, an example as disclosed herein being the ONC-T18 strain which has an ATCC strain deposit accession number of PTA-6245.

63. It is understood that the oil producing microbes and any clones, modified organisms or genes isolated from said organism as set forth in ONC-T18 are also disclosed. The disclosed the oil producing microbes have the ability to produce unsaturated fatty acids, such as lipids containing the omega-3 series of DHA, EPA and DPA, and the omega-6 series of DPA and various antioxidant such as carotenoids, xanthophylls and phenolics.

64. Also disclosed are processes for the production of biomass containing said compounds. Further disclosed are processes for preparing omega-3, omega-6 and

carotenoid compounds utilizing the the oil producing microbes. Also disclosed are processes for the production of microbial derived (or single celled) oils.

65. In addition, disclosed are the fatty acids and carotenoids produced by the disclosed oil producing microbe and any progeny (genetically modified or otherwise), various feedstuffs, nutraceuticals, pharmaceuticals and foods supplemented with the lipids and antioxidants, as well as a process for utilizing these compounds as an additive for various feedstuffs and foods.

66. U.S. Patent No. 5,130,242 to Barclay disclosed a collection and screening process to isolate strains of microorganisms with the following characteristics for the production of omega-3 fatty acids: 1) capable of heterotrophic growth; 2) produce a high content of omega-3 fatty acids; 3) unicellular; 4) produce a low content of standard and omega-6 fatty acids; 5) non-pigmented, white or colorless cells; 6) thermotolerant (e.g., ability to grow above 30°C); and 7) eurhaline (e.g., ability to grow at a wide range of salinities, but preferably at low salinity).

67. The '242 disclosure also describes a process for the heterotrophic production of whole-celled or extracted microbial products with a high concentration of omega-3 fatty acids, which can later be used in animal or human food products. This process uses microorganisms identified by the collection and screening process disclosed thereof. These microorganism, which are of the order Thaustochytriales, are cultured in ground grain. To enhance production of omega-3 fatty acids, low temperature stressing and high dissolved oxygen are used, as well as the addition of antioxidants, growth factors, vitamins, and phosphorous. The extracted products contain high concentrations of omega-3 fatty acids (e.g., C20:5w3, C22:5w3; and C22:6w3) and low concentrations of omega-6 fatty acids (e.g., C20:4w6 and C22:5w6). Specifically, the ratios of the C20:5w3 to C22:6w3 fatty acids run from 1:1 to 1:30. Ratios of C22:5w3 to C22:6w3 fatty acids run from 1:12 to only trace amounts of C22:5w3. Also, the microorganisms produce from 0.6 to 0.72 % DHA, 0 to 5 % DPA, and 0 to 18.9 % EPA, by weight of total fatty acid.

68. U.S. Patent No. 6,451,567 to Barclay disclosed a process for growing *Thraustochytrium* and *Schizochytrium* in a non-chloride medium (<3g/L) containing sodium salts (e.g., sodium sulfate). The non-chloride medium results in cell aggregate sizes of less than 150 μm. The disclosed process produces microorganisms and extracts that are useful in food products for aquaculture. Further components of the food products include flaxseed, rapeseed, soybean, and avocado meal. The

microorganisms can produce up to 1.08 g of omega-3 fatty acids per liter of medium per day. The '567 disclosure further describes various culture mediums, which include sea water, glucose (1, 3, 5, or 10 g/L), yeast extract (0.01, 0.2, 0.4 and 5 g/L), additional nitrogen sources such as protein hydrosylate (1 g/L), liver extract (1 g/L), glutamate (5 g/L), mono-sodium glutamate (MSG) (3 g/L), and additional salts, trace vitamins and minerals (e.g., KH_2PO_4, $MgSO_4$, and gelatin extract).

69. U.S. Patent No. 6,582,941 to Yokochi et al. discloses a *Schizochytrium* species, strain SR21 and another *Schizochytrium* strain belonging to the same species that have the ability to produce fatty acid fractions having a high concentration of omega-3 DHA and/or omega-6 DPA and a low concentration of EPA. Also, disclosed are methods of culturing such microorganisms and isolating such fatty acids. The medium used contains sea salt, yeast extract (0.2, 1.0, or 10 g/L), corn steep liquor (0.5, 1.0, or 10 g/L), glucose (10-120 g/L), plus additional salts (e.g., NH_4OAc, phosphates). The fatty acid compositions contain about 15 to 20% DHA by weight of biomass (about 28% by weight of total fatty acid). The compositions can be used in food products (e.g., baby milk).

70. U.S. Patent No. 6,607,900 to Bailey et al. disclosed a process for growing eukaryotic microorganisms (e.g., *Schizochytrium* sp. ATCC 20888) that are capable of producing at least 20% of their biomass as polyunsaturated lipids (particularly omega-3 and -6 fatty acids). The process involves culturing the microorganisms in a medium containing a carbon and nitrogen source. Also disclosed is the use of low dissolved oxygen levels (less than 3%) and low chloride ion levels (less than 3 g/L) to enhance production. The microorganisms have a lipid production rate of at least 0.5 g/L/h. The lipid fraction is from 15 to 20 % DHA by weight of biomass (about 35% by weight of total fatty acid methyl esters).

71. U.S. Publication No. 2004/0161831 to Komazawa et al discloses a *Thraustochytrium* strain (LEF1; accession number FERM BP-08568 at the Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Japan) that has the ability to produce DHA. By culturing in conventional media, the microorganism can produce oil with at least 50% by weight DHA. The oil can be treated with a lipase prior to isolation of DHA. The oil can be used in food or drinks or the DHA can be hydrolyzed to produce behenic acid.

1. Fatty acids

72. Fatty acids are hydrocarbon chains that terminate in a carboxyl group, being termed unsaturated if they contain at least one carbon-carbon double bond, and polyunsaturated when they contain multiple such bonds. Long-chain polyunsaturated fatty acids (PUFA) or highly-unsaturated fatty acids (HUFA), may be divided into the (n-3) and (n-6) series as a result of the location of these double bonds. There is overwhelming scientific evidence that (n-3) highly unsaturated fatty acids such as DHA have a positive effect on cardio-circulatory diseases, chronic inflammation and brain disorders. The (n-6) fatty acids on the other hand have been noted as intermediate metabolites within the eicosanoid steroids, such as prostaglandins, leucotrienes or the like.

73. Polyunsaturated fatty acids can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 double and/or triple carbon-carbon bonds. For example, polyunsaturated fatty acids can comprise 3-8, 4-7, or 5-6 double and/or triple carbon-carbon bonds.

74. Currently, the main source of these highly unsaturated fatty acids is fish, with EPA and DHA noted within various fish species (such as sardines, anchovies, pilchards and tuna) at amounts around 20% and 10%, respectively. Yet, if one intends to use fish oil as the sole source of these lipids, several disadvantages exist, such as problems with flavor taint, uncontrollable fluctuations in availability, natural fish oil content variability, as well as the potential to accumulate harmful environmental pollutants if not processed correctly. In addition, if one intends to obtain a highly purified (n-3) or (n-6) oil from said sources, it is very difficult to preferentially separate and purify the desired oils without extensive processing. Specifically, a large market is available within the neonatal supplement market for a highly concentrated form of DHA. If fish oil were to be the source of said products, then DHA would have to be preferentially isolated in large quantities from EPA. Clearly an alternative source of highly purified and production/or customer tailored source of these highly unsaturated fatty acids is needed. One such source is the use of fatty acid production antagonists which affect directly the lipid metabolic pathway or those associated with fatty acid production, such as the carotenoid pathway which is produced in parallel with lipids for their antioxidant properties in oil producing microbes.

75. Shirasaka et al. (2005) Mycoscience 46:358-363 used both cyanocobalamin and p-toluic acid to modify the fatty acid composition of *Schizochytrium limacinum* SR21, by up-regulating the cobalamin-dependant methylmanoyl-CoA mutase

responsible for the conversion of propionic to succinic acid in the case of cyanocobalamin (an active component of vitamin B_{12}) and by reducing the omega-6 DPA concentration and increasing the EPA concentration in a dose dependant manner, with respect to p-toluic acid supplementation.

76. In addition to fish oils, various microorganisms (mainly marine) are able to produce and/or accumulate the (n-3) series of docosahexaenoic acid. Of particular interest is the fact that microbial production is not subject to fluctuations caused by external variables such as seasonality, weather and food supply. For example, the following oil producing microbes are known as having the ability to produce DHA: the deep-sea derived bacterium *Vibrio marinus* (ATCC 15381), *Vibrio* sp. T3615, *Photobacterium profundum* SS9, *Mortierella marina* MP-1 and *Psychromonas kaikoae* (ATCC BAA-363T); microalgal species such as *Crypthecodinium cohnii*, *Cyclotella cryptica*, and *Mortieralla alpina* (1S-4); and the protists *Thraustochytrium* sp. (ATCC 20892), *Thraustochytrium aureum* (ATCC 34304) and *Thraustochytrium roseum*. According to a process utilizing these purified organisms, however, the amount of arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid produced per gram of biomass per liter is low, being within the range of 10 to 500 mg. Some examples and representative microbial oil producing organisms are shown in Figure 5.

77. Omega 3s have been shown to have beneficial effects and the oils and compositions disclosed herein can be used for anti-inflammatory effects on cystic fibrosis (Cochrane Database Syst Rev. 3), rheumatoid arthritis (Drugs 63: 845-53), asthma (Ann Allergy Asthma Immunol 90: 371-7) and thrombotic stroke (Prev Cardiol 6: 38-1), cardio-protective, as well as a direct effect on artheriosclerosis and arrhythmia (Prostaglandins Leukot Essent Fatty Acids. 63:351-62), inhibition of cancer proliferation in breast and prostate cancer cell lines and reduction in animal experiments (Am J Clin Nutr 77: 532-43), anti-psychotic effect on schizophrenia (J Neural Transm Suppl 64:105-17) and other psychiatric diseases (Can J Psychiatry 48: 195-203), immunonutrient supplement used for normal neonatal development and in the treatment of neonatal infections (Eur J Pediatr 162: 122-8), and pathological pain treatment by directly attenuate the neuronal and ganglial processes that underlie neuropathic and inflammatory pain (Prostaglandins Leukot Essent Fatty Acids 68: 219-24).

2. Thraustochytriaceae

a) ONC-T18

78. An example of an oil producing microbe is the marine micro-organism ONC-T18. The marine micro-organism ONC-T18, as disclosed herein, was collected as part of a PUFA producing microbial isolation trip, with over 60 pure cultures isolated (see table 7 for details). Further, ONC-T18 was isolated from the leaves of salt marsh grasses in the Advocate Harbor area, Bay of Fundy, Nova Scotia, Canada. Through microscopic examinations, serial culture purification techniques, and DNA sequencing techniques, the strain was found to be a single micro-organism belonging to the genus *Thraustochytrium*. All strains and two ATCC comparison cultures (ATCC 20891 & MYA-1381) were grown on 0.5% glucose, 0.2% peptone, 0.2% yeast extract in sea-water (SW) and underwent gas chromatography (fatty acid methyl ester, FAME) analysis.

79. Figure 6 shows a proposed phylogenetic, branch tree diagram of the relationship between ONC-T18, and other members of the order *Labyrinthulida*, based upon comparison of sequences obtained through the use of the highly conserved 18S ribosomal RNA gene.

80. Figures 4 shows a phylogenetic trees developed using Neighbour-joing and bootstrap analysis techniques in order to determine the relationship of ONC-T18 with other members of the family *Thraustochytriidae*, again using the 18S ribosomal RNA gene directly sequenced and compared to other sequences within the public domain using similarity matrices.

81. ONC-T18 was originally isolated using classic pine pollen baiting techniques, followed by culturing on selective medium. Specifically, a nutrient medium containing 5 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} yeast extract to 1 L of 0.2 μm filtered sea water was prepared. The fatty acid profile of ONC-T18 was then determined using the Bligh and Dyer extraction method and PUFA gas chromatographic techniques. Chromatographic results demonstrated the ability of this strain to produce increased amounts of total fatty acids (TFA), DHA, as well as marked quantities of EPA and DPA (both n-3 and n-6 fatty acids).

82. The disclosed oil producing microbes, including ONC-T18, can be used in a process for preparing a lipid or fat containing DHA, EPA and DPA, but is not limited to the above-mentioned ONC-T18 or PTA-6245 strain, but any derivation of said strain whether it be via genetic modification, chemical mutagenesis, fermentative

adaptation or any other means of producing mutants of the strain, whereby the product of these modifications have genetic, morphological and/or functional features such as the oil producing microbes, as disclosed herein.

83. Disclosed are oil producing microbes capable of producing a lipid composition having distinctive lipid class properties, and the solution to the problem of maintaining a stable, reliable and economical source for such a lipid having high functionality and additional value according to the same. Therefore, wild-type strains producing the (n-3) series of DHA and the (n-6) series of DPA to a greater degree as well as variant and recombinant strains designed to produce these polyunsaturated fatty acids to a greater degree are disclosed herein. Such variant or recombinant microorganisms include those designed to have a higher content of said lipids than those produced by the original wild-type strain, when cultured using the same conditions and media. In addition, microorganisms designed to produce a lipid containing similar amounts of the (n-3) series of DHA, EPA and the (n-6) series of DPA can be selected, as compared with the corresponding wild-type strains, effectively using substrates having a superior cost performance, are also included.

84. Also disclosed are compositions comprising oil producing microbes of the order Thraustochytriales wherein the oil producing microbe produces unsaturated fatty acids. The polyunsaturated fatty acids can be, for example, omega 3 or omega 6 fatty acids, such as DHA and DPA.

85. Also disclosed are compositions comprising one or more oil producing microbes of the order Thraustochytriales wherein the oil producing microbe(s) produce specific fatty acids when their growth is supplemented with fatty acid production antagonists.

86. Also disclosed are compositions comprising one or more oil producing microbes of the order Thraustochytriales wherein the oil producing microbe(s) produce elevated amounts of specific unsaturated fatty acids, for example omega-3 fatty acids, such as EPA by supplementing standard fermentative media with one or more fatty acid production antagonists. Use of oleic acid in such a manner is illustrated in Figure 9 and example 14(c).

87. Disclosed are compositions comprising a oil producing microbe, wherein the composition produces a lipid.

88. Also disclosed are compositions comprising a oil producing microbe(s), wherein the composition produces a lipid, wherein the lipid comprises a lipid as disclosed herein.

89. Also disclosed are compositions comprising one or more oil producing microbes of the order Thraustochytriales wherein the oil producing microbe(s) produce ketocarotenoid compounds. Ketocarotenoid compounds include, but are not limted to carotenes and xanthophylls, such as β-carotene and astaxanthin.

90. Carotenoids are naturally occurring lipid soluble compounds, synthesised from the ubiquitous C_5 isopentenyl pyrophosphate precursor, and its structural isomer dimethyallyl pyrophosphate. Carotenoids compose an abundant, highly diverse group of compounds. Within this group of compounds two classes exist, the hydrocarbon carotenoids, carotenes, and oxygenated derivatives of carotenes, xanthophylls.

91. Carotenoids are also one of the most abundant and diverse groups of naturally occurring compounds on earth, being responsible for the vibrancy of the world in which we live. Traditionally, carotenoids have been used in the feed, food and nutraceutical industries. They are produced ubiquitously by photosynthetic organisms, including cyanobacteria, but are also wide spread among non-photosynthetic bacteria and fungi (Goodwin, *The Biochemistry of Carotenoids.* New York: Chapman and Hall (1980)). Although carotenoids are classed as secondary metabolites, they are known to be vital for plant growth and photosynthesis. Carotenoids are also essential to many higher organisms, including humans, many of which are not able to synthesise these compounds. Thus dietary intake is the sole source of carotenoids for many organisms. As a result research into dietary sources, and actual consumption of carotenoids, has received much interest in recent years. In 2006, the global market for carotenoids was estimated to be worth US$ 954.7 million, with expectations for this to increase to US $1069.2 million by 2010 (Carotenoids: A Global Strategic Business Report, 2006).

92. One role of dietary carotenoids is that of a precursor to vitamin A (retinoids). Significant as vitamin A deficiency is the leading cause of preventable blindness in children, while also increasing the risks of disease and death from sever infection (WHO). As dietary antioxidants, carotenoids such as β-carotene, lycopene, astaxanthin, canthaxanthin and lutein, exhibit significant anti-cancer activities, and play an important role in the prevention of chronic diseases (Edge, *B-Biology* 4, 41, 189-200 (1997); Singh, *Oncology-New York*, 12, 1643 (1998); Smith, *British Journal*

of Biomedical Science, 55, 268-275 (1998)). Further, consumption of increased amounts of carotenoids has been related to reduced occurrences of degenerative disease such as age-related macular degeneration, the main cause of age related blindness, as well as some cancers and chronic heart disease (Basu, *JAOCS*, 78 (7), 665-675 (2001); Mares-Perlman et al., *J Nutr*, 132 (3), 518S-524S (2002)).

93. Owed to their backbone of conjugated double bonds, carotenoids are potent biological antioxidants that can absorb the excited energy of singlet oxygen onto the carotenoid chain, leading to the degradation of the carotenoid molecule, but preventing other molecules or tissues from being damaged (Schagerl and Muller, *J Plant Physiol* 2005). Carotenoids can be divided into two structurally distinct classes, the carotenes and xanthophylls. Carotenes, possess a hydrocarbon backbone, while xanthophylls contain one or more oxygen-containing functional group (Armstrong and Hearst, *Faseb J*, 10 (2), 228-3 (1996); Armstrong, *Annu Rev Microbiol*, 51, 629-59 (1997)). Carotenes are common to many organisms, with a highly conserved mode of biosynthesis. Xanthophylls on the other hand, are produced by a small clutch of organisms. Final products and routes of synthesis are often species specific. Of the Carotenoids, it is the xanthophylls that attract most interest, specifically β-carotene, canthaxanthin and astaxanthin. With β-carotene demanding the largest portion of the global market, while canthaxanthin and astaxanthin command 14.92% and 21.58% of the market, respectively (Carotenoids: A Global Strategic Business Report, 2006).

94. The xanthophylls encompass various compounds such as the ketocarotenoids, echinenone and canthaxanthin, synthesised by the addition of one or two carbonyl groups at the 4 and 4` positions of β-inone ring of β-carotene, respectively. A reaction catalysed by the class of genes collectively known as β-carotene ketolase genes. β-crytpoxanthin and zeaxanthin are also classed as xanthophylls, these are synthesised by the direct replacement of a hydrogen atom at the 3 and 3` positions of the β-ionone ring of β-carotene with a hydroxyl group (Tian and DellaPenna, *Arch Biochem Biophys*, 430 (1), 22-9 (2004)). Additionally, there exists a plethora of combined keto and hydroxylated carotenoids, such as 3-hydroxyechinenone, 3`-hydroxyechinenone, adonixanthin, and adonirubin. All compounds listed here are valuable compounds in their own right with important properties, but significantly they constitute important precursors for the biosynthesis of astaxanthin. Astaxanthin is the final product of this pathway, being both hydroxylated, and ketolated at the 3, 3` and 4,4` carbons of the β-ionone rings of the β-carotene

skeleton. Yet the actual pathway from β-carotene to astaxanthin is not well characterised, and may prove to be species specific (Tao et al., *Metab Eng.* (2006)).

95. Also disclosed are compositions comprising an oil producing microbe, wherein the composition produces an antioxidant.

96. Also disclosed are compositions, wherein the oil producing microbe comprises a member of the order Thraustochytriales.

97. Also disclosed are compositions, wherein the oil producing microbe has a 18S ribosomal RNA gene sequence having at least 80% identity to SEQ ID NO:1.

98. Also disclosed are compositions, wherein the oil producing microbe has a 18S ribosomal RNA gene sequence having at least 80% identity to GenBank accession number DQ374149.

99. It is understood that any form of characterization described herein, such as by genetics or by the lipid signatures or by the classifications, for the oil producing microbes can be used to characterize the oil producing microbes as disclosed herein. The oil producing microbes can comprise one or more microorganisms from the family Thraustochytriaceae, and examples are ATCC accession number 20888, 20889, 20890, 20891, 20892 and PTA-6245. There are a variety of characteristics that can be used related to the organisms and the unsaturated fatty acids or carotenoids they produce. It is understood that these can be used in any combination or permutation to define a set or sets of organisms or oils or antioxidants, for example. One characteristic is the classification of the organisms themselves, the genetic identification of the organisms, the lipid and antioxidant profiles of the organisms, and the growth conditions of the organisms, for example.

b) Classification

100. The oil producing microbes that can be used in the methods described herein can be from the phylum Labyrinthulomycota. The oil producing microbes can be from the class Labyrinthulomycetes. The oil producing microbes can be from the subclass Thraustochytridae. The oil producing microbes can be from the order Thraustochytriales. The oil producing microbes can be from the family Thraustochytriaceae. The oil producing microbes can be from the genus *Thraustochytrium*. The oil producing microbes can be a *Thraustochytrium* sp. The oil producing microbes can be *Thraustochytrium aureum*. The oil producing microbes can be *Thraustochytrium roseum*. The oil producing microbes can be *Thraustochytrium striatum*. The oil producing microbes can be from the genus

Schizochytrium. The oil producing microbes can be *Schizochytrium* sp. The oil producing microbes can be a modified version of any of the listed oil producing microbes. The oil producing microbes can also comprise any currently unknown or isolated members of said prokaryotes class, subclass, order, family or genus. A combination of oil producing microbes an be any combination of any oil producing microbes disclosed herein, including, one or more of the *Thraustochytrium* sp., *Schizochytrium* sp., *Thraustochytrium aureum*, *Thraustochytrium striatum* and *Thraustochytrium roseum*.

101. The oil producing microbes from the family Thraustochytriaceae can be any of those disclosed above. The oil producing microbes can comprise the organism having ATCC accession number PTA-6245.

c) Genetics

102. The oil producing microbes can have 18S rRNA sequence SEQ ID NO:1. The oil producing microbes can have 18S rRNA sequence described in GenBank accession number DQ374149. The oil producing microbecan have an 18S rRNA sequence that, for example, has about 90% homology, or any other identity disclosed herein, to SEQ ID NO:1. The oil producing microbe can have an 18S rRNA sequence that hybridizes under stringent conditions, or any other conditions as disclosed herein, to SEQ ID NO:1, or a portion of SEQ ID NO:1.

103. The sequence similarity/identity and nucleic acid hybridization of the nucleic acids of the organisms can be as described herein. Specifically, comparison of SEQ ID NO:1 with nucleic acid sequences found in the genomic database, GenBank (National Centre for Biotechnology Information, National Institute of Health, Bethesda, MD, USA) using the BLAST (Basic local alignment search tool) algorithm identified SEQ ID NO:1 as being related (91% similarity) to several eukaryotic Thraustochytrid species, closely related to *Thraustochytrium* sp. CHN-1 [AB126669] (94.5% similarity) and *Thraustochytriidae* sp. N1-27 [AB073308] (95.5% similarity), and most closely related to *Thraustochytrium striatum* [AF265338] (97.5% similarity).

3. Plants

104. Fatty acid production antagonists can also be used to influence oil production ratios in oil producing plants, including both transgenic and naturally occurring species and include representative crops such as rapeseed, flux seed, soybean and sunflower. Methods of incorporation of said antagonists include, for example, watering into areas surrounding plants for incorporation in the plants via the

root systems, addition as an active ingredient in sprays for direct incorporation into the plant cells. Additionally direct immersion, vaporization or injection of these compounds into the plant cells via either the stomata or subcutaneously may also be considered an effective method.

4. (1) Sequence similarities

105. It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

106. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of nucleic acids and proteins herein disclosed typically have at least, about 50, 55, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

107. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2:482, 1981, by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970, by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

108. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

109. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

(2) Hybridization/selective hybridization

110. The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face

or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

111. Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6x SSC or 6x SSPE) at a temperature that is about 12-25°C below the T_m (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5-20°C below the T_m. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6x SSC or 6x SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

112. Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example,

in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed under conditions where both the limiting and non-limiting primer are for example, 10, 100 or 1000 fold below their k_d, or where only one of the nucleic acid molecules is 10, 100 or 1000 fold or where one or both nucleic acid molecules are above their k_d.

113. Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 50, 55, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 50, 55, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

114. Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example, if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

115. It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

d) Composition of molecules produced

116. It is understood that the oil producing microbes disclosed herein are capable of producing a number of compounds and compositions. The compounds and

compositions can be used as a signature, a way of identifying the oil producing microbes. For example, one way of characterizing an oil producing microbe is by the lipid profile that the oil producing microbe produces. As disclosed herein these various lipid profiles can be used to characterize the oil producing microbes as well as be purified, manipulated, and collected for a variety of reasons.

(1) Lipids

117. It is understood that each oil producing microbe can produce some profile of unsaturated fatty acids, as disclosed herein. These profiles are characteristics of the oil producing microbes. Below are some examples, of unsaturated and other lipid profiles for the oil producing microbes.

118. The oil producing microbe can produce, for example a lipid or fatty acid fraction of at least about 4 wt.% to 6 wt.% (e.g., about 5 wt.%), which comprises from about 0 wt. % to about 2 wt. % myristic acid (e.g., about 1 wt.%), from about 16 wt.% to about 20 wt.% (e.g., about 18 wt.%) palmitic acid, from about 0 wt. % to about 2 wt.% (e.g., about 1 wt.%) palmitoleic acid, from about 4 wt.% to about 8 wt.% (e.g., about 6 wt.%) stearic acid, from about 30 wt.% to about 34 wt.% (e.g., about 32 wt.%) oleic acid, from about 40 wt.% to about 44 wt.% (e.g., about 42 wt.%) linoleic acid, and from about 0 wt.% to about 3 wt.% (e.g., about 2 wt.%) n-3 EPA per dried cellular biomass.

119. The oil producing microbe can also produce, for example, a lipid or fatty acid fraction of at least about 1 wt.% to 3 wt.% (e.g., about 1.25 wt.%), which comprises from about 2 wt.% to about 4 wt.% (e.g., about 3 wt.%) myristic acid, from about 50 wt.% to about 60 wt.% (e.g., about 55 wt.%) palmitic acid, from about 2 wt.% to about 4 wt.% (e.g., about 3 wt.%) palmitoleic acid, from about 16 wt.% to about 20 wt.% (e.g., about 18 wt.%) stearic acid, from about 9 wt.% to about 13 wt.% (e.g., about 11 wt.%) oleic acid, from about 1 wt.% to about 3 wt.% (e.g., about 2 wt.%) eicosadienoic acid, and from about 6 wt.% to about 10 wt.% (e.g., about 8 wt.%) n-3 EPA per dried cellular biomass.

120. The eukaryotic microorganism, for example, such as ONC-T18, can produce at least about 30 wt.%, 40 wt.%, 50 wt.%, 60 wt.%, 70% wt.% or 80 wt.% (e.g., about 80 wt.%) of a lipid composition per dried cellular biomass. For example, the oil producing microbe can produce a lipid composition comprising from about 25% to about 40% of an omega-3 fatty acid, such as n-3 DHA, (for example, at least 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55% or 60% by weight), and from

about 0% to about 3% of the omega-3 fatty acid, EPA, (for example, at least 1% or 2% by weight) and from about 4% to about 12% of an omega-6 fatty acid, such as n-6 DPA, (for example, at least 4%, 5%, 6%, 7%, 8%, 9%, or 10% by weight).

121. It is understood that the composition of the lipid produced by the oil producing microbe can be manipulated based on the growing conditions the oil producing microbe multiplies in. By changing various parameters, as disclosed herein, the compositions can be manipulated to produce, for example a better yield of DHA, EPA or DPA. For example, the manipulation may not produce more actual grams, but the manipulation, may produce a better ratio of DHA or DPA to EPA and other desired PUFAs, which may be desirable from a purification standpoint. Varying conditions that can be used to increase the yield of DHA, EPA or DPA are also discussed herein.

122. It is understood that the composition of the lipid produced by the oil producing microbe can be manipulated based on the growing medium composition the oil producing microbe multiples in. By changing both the ratios and types of carbon to nitrogen sources, the chemical composition of salt used and by the addition of certain chemical compounds which impact upon fatty acid production, specific fatty acid profiles similar to those of selected fish oils may be produced. For example, the manipulation may not produce more actual grams, but the manipulation, may produce a TFA profile similar to that of, for example, cod liver oil, tuna or bonito oil or classical 18:12 fish oil.

123. Disclosed herein are methods of altering the fatty acid ratios produced by oil producing microbes by supplementing the growing medium compositon that the oil producing microbe multiplies in with one or more fatty acid production antagonists. The fatty acid ratios can be altered with or without altering the overall fatty acid content of the oil producing microbe. For example, the ratio of omega n-3 and/or n-6 oils to omega n-9 oils can be increased by summplementing the medium of an oil producing microbe with a fatty acid production antagonist.

124. Also disclosed are methods of altering the fatty acid ratios produced by oil producing microbes without altering the overall fatty acid content of the oil producing microbe, by supplementing the growing medium compositon the oil producing microbe multiplies in with one or more fatty acid production antagonists.

125. Figure 1 shows an example of a metabolic pathway for the various PUFAs produced by the disclosed eukaryotic microorganism, consistent with fatty acid methyl

ester metabolite tracking. Proteins which can be identified within the pathways which as disclosed herein are polyketide synthase, using for example a degenerative primer study, (Metz et al. (2001) Science 293:290-3 and Kaulmann & Hertweck (2002) Angew. Chem. Int. Ed. 41:1866-9). Also elongases and desaturases, using for example a hybridization probe and/or degenerative or targeted primers can be identified. Also fatty acid synthases can be identified using, for example, a hybridization probe and/or a degenerative primers.

5. Growing and culturing

126. A phenotypic microplate study including carbon; nitrogen (peptide nitrogen); phosphorus and sulfur; osmolytes, and pH was performed.

127. An Orthogonal array (Taguchi) method was used to determine optimum media configurations and variations in nitrogen, carbon and salt concentration (Joseph J & Piganatiells JR (1998) IIE Trans, 20:247-254).

128. When fermenting ONC-T18, it was found that if you increase agitation or dO_2 you increase biomass production & TFA but decrease DHA. If you decrease agitation or dO_2 you decrease cellular biomass (g) & decrease TFA but increase DHA but also reduce C16:0, C16:1 & C18:1.

129. If you increase temperature you increase biomass production & TFA but decrease DHA. If you decrease temperature you decrease cellular biomass (g) & decrease TFA but increase DHA, but reduce C16:0, C16:1 & C18:1.

130. Cellular biomass derived from the disclosed oil producing microbecan be obtained by inoculating a suitable natural or artificial seawater medium, containing from about 2 % to about 100% of seawater. This oil producing microbeis able to utilize various nutritional components within this medium. Examples of a carbon source used within the medium are carbohydrates such as glucose, fructose, dextrose, lactulose, galactose, maltotriose, maltose, lactose, glycogen, gelatin, starch (corn or wheat) as well as sugar derivatives such as acetate, m-inositol (derived from corn steep liquor), galacturonic acid (derived from pectin), L-fucose (derived from galactose), gentiobiose, glucosamine, α-D-glucose-1-phosphate (derived from glucose), cellobiose (derived from cellulose) dextrin (derived from corn) and α-cyclodextrin (derived from starch) and polyols such as maltitol, erythritol, adonitol and oleic acids such as glycerol and tween 80 and amino sugars such as N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine. While, examples of a nitrogen source are natural nitrogen sources such as peptone, yeast

extract,malt extract and fish meal, or an organic nitrogen sources such as sodium glutamate, but not limited thereto. Furthermore, if necessary phosphates, such as potassium phosphate, and sodium phosphate, inorganic salts such as ammonium sulfate, sodium bicarbonate, sodium orthovanadate, potassium chromate, sodium molybdate, selenous acid, nickel sulfate, copper sulfate, zinc sulfate, cobalt chloride, iron chloride, manganese chloride and calcium chloride may be used as trace nutrients, along with the chelating compound, ethylenediaminetetraacetic acid, alone or in conjunction with vitamins such as pyridoxine hydrochloride, thiamine hydrochloride, calcium pantothenate, p-aminobenzoic acid, riboflavin, nicotinic acid, biotin, folic acid and vitamin B_{12}. After preparing the medium, the pH is adjusted to between 3.0 and 10.0 using acid or base to adjust where appropriate, for example between pH 4.0 and 6.5, and the medium is sterilized by autoclaving, for example. Cultivation can be carried out for 1 to 30 days, 1 to 21 days, 1 to 15 days, 1 to 12 days, 1 to 9 days, or preferably 3 to 5 days at temperatures between 4 to 30°C, preferably 18 to 28°C, by aeration-shaking culture, shaking culture, stationary culture, batch culture, continuous culture, rolling batch culture, or wave culture, or the like.

131. The following conditions are an example of conditions that can allow for the production of a set of lipids in yields which allow for their use as a commodity. Investigation of culture conditions for ONC-T18 revealed that the oil producing microbe disclosed herein grows well in natural or artificial sea water or in a medium containing down to 5% concentration of natural or artificial sea water. Carbon and nitrogen sources added to the medium may be those conventionally used as described above. The nitrogen source being either natural or organic in nature is relatively equal and total nitrogen concentration within the medium is kept constant. These sources are added to the medium at standard concentrations. If these conditions are met, little influence on the content of lipid, proportions or the amount of accumulated DHA, DPA and EPA is produced, as disclosed herein.

132. For high-concentration fermentation of ONC-T18, it is possible to use several methods to increase both cellular biomass and lipid production rates. These include, increasing both the carbon and nitrogen concentration in the medium (at a ratio of between 6:1 and 15:1, preferably between 6:1 and 13:1 and at temperatures between 4 to 30°C, preferably 18 to 28°C) from the range 5 g L^{-1} to 60 g L^{-1} to the range 100 g L^{-1} and 160 g L^{-1} and from the range 4 g L^{-1} to 10 g L^{-1} to the range 40 g L^{-1} to 60 g L^{-1}, respectively. Using this method the proportion of biomass and lipid

produced is also increased at comparable rates. Furthermore, it is possible to increase lipid production through the use of increased carbon sources from the range 5 g L^{-1} to 60 g L^{-1} to the range 100 g L^{-1} and 160 g L^{-1}, while the nitrogen source remains constant. Additionally, it is possible to increase biomass production while maintaining lipid content, through the use of increased amounts of nitrogen sources from the range 10 g L^{-1} to 60 g L^{-1} while the carbon source remains constant. Moreover, experimentation has determined that biomass and lipid production greatly increases with increased agitation from the range 100 and 1000 rpm, better at between 350 and 600 rpm and optimal at between 350 and 450 rpm, with only a marginal decrease in lipid content and no decrease in fatty acid profiles, with agitation particularly relevant at the early stages of heterotrophic fermentation. Experimentation has also determined that lipid production optima are achieved when the dissolved oxygen content of the culture medium is held between 1 and 10%, optimally at 5% during the lipid accumulation stage of classical fed-batch fermentation of these micro-organisms (See Figure 8). Finally, the addition of acetate, trace elements, metals and vitamins to the production medium (as mentioned above) increases the production of DHA, EPA and DPA with respect to other fatty acids without decreasing total lipid values.

133. By performing heterotrophic fermentation as described above, it is possible to consistently produce cellular biomass which produces a lipid containing the (n-3) series of DHA in a culture of high concentration of not less than 5 g and more preferably not less than 20 g/L medium (see Figure 8). Furthermore, experimentation has shown that most of these lipids accumulate during the later-exponential/transition stages of cultivation, after maximal biomass levels are reached. Yet, during the fermentation process, lipid content typically does not fall below 25% of the total biomass, typically maximizing at around 80%. Cultivation under the above conditions can be carried out using a conventional agitation-fermenter. It is also possible to use a bubble column fermenter (batch or continuous cultures), or a wave fermentor.

134. Collection of cellular biomass prior to processing for lipid separation can be performed using various conventional methods such as centrifugation (such as solid-ejecting centrifuges) or filtration (such as cross-flow filtration) and may also include the use of a precipitation agent for the accelerated collection of cellular biomass (such as sodium phosphate, calcium chloride or polyacridamide).

a) Fatty Acid Production Antagonists

135. The medium used to grow the disclosed oil producing microbes can be also comprise fatty acid production antagonists. Fatty acid production antagonist can be fatty acid subunits or fatty acid pathway modulators. For example, fatty acid production antagonists include, but are not limited to, oleic acid, cerulenin, toluic acid, curcumin, cyclopropene, alkyl gallate, propyl gallate, sesame oil, peanut oil, canola oil, pyridazinone, norflurazon, diphenylamine, sethoxydim, and capsaicin.

136. The term "fatty acid subunit" or "fatty acid subunits" as used herein refers to the 16, 18, 20, 22 or 24 carbon fatty acids involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. For example, "fatty acid subunits" can include any of the 16, 18, 20, 22 or 24 carbon fatty acids as shown in Figure 1.

137. A "fatty acid subunit" can also be used herein to refer to any 16, 18, 20, 22 or 24 carbon fatty acids having 1, 2, 3, 4, 5 or 6 unsaturations involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. Further embodiments can include odd numbered carbon fatty acids, with or without unsaturations such as 15, 17, 19 and 21 carbon fatty acids found within marine organisms.

138. Fatty acid pathway modulators can be compounds capable of inhibiting one or more of the enzymes involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. Inhibition of one or more of the enzymes involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes can be, unless otherwise indicated, at least 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% inhibition.

139. A fatty acid pathway modulator can be a compounds capable of inhibiting one or more of the desaturases or elongases involved in the metabolic pathway for the production of PUFAs for the disclosed oil producing microbes. Fatty acid pathway modulators can also be compounds capable of inhibiting phytone desaturases, β-carotene C4-oxygenases, fatty acid synthases or acetyl CoA carboxylases of the disclosed oil producing microbes.

6. Isolation of Lipid

140. Figure 2 shows a general fatty acid profile for a disclosed oil producing microbe. Figures 5-7 show biomass (g/L), lipid (TFA) and DHA content (both mg/g biomass) as a function of fermentation in shake flasks on a variety of nitrogen sources both singularly or in the presence of L-glutamate or yeast extract (YE), carbon sources (singularly) or cultures supplemented (i.e. in addition to both nitrogen and carbon sources mentioned throughout) with various antioxidant, phytohormone or simple carbon sources. All of this data can be used to extract specific characteristics about the disclosed oil producing microbes.

141. The fat containing the (n-3) series of DHA, DPA, EPA and the (n-6) series of DPA can be obtained by breaking or disrupting the collected cell biomass, for example, via milling, ultrasonication, and then carrying out extraction with a solvent such as chloroform, hexane, methanol, ethanol or via supercritical fluid extraction means. The content of the resultant fat containing the (n-3) series of DHA and the (n-6) series of DPA per gram of dried cellular biomass is preferably greater than 0.25 g and more preferably greater than 0.6 g.

142. The disclosed oil producing microbes, including the oil producing microbe, ONC-T18, are able to produce lipid thus obtained, any of its variants and any associations between members of the same species of eukaryotic microorganisms whereby the lipid profile is as follows. The percentage of neutral lipids can be at least 95% by weight of total lipids. For example, the typical composition of fatty acids for ONC-T18, in the neutral lipids is as follows: 15% of myristic acid, 8% of pentadecanoic acid, 35% of palmitic acid, 7% of palmitoleic acid, 1% of stearic acid, 2% of oleic acid, 1% of eicosapentaenoic acid, 6% of decosapentaenoic acid and 25% docosahexaenoic acid. Upon the supplementation of the medium with one or more fatty acid production antagonists, the overall ratio of omega n-3 and omega n-6 oils such as docosahexaenoic acid, eicosapentaenoic acid and decosapentaenoic acid (both n-3 and n-6) to the omega n-9 oils such as stearic and can be increased. For example, Figure 11 shows possible changes in the ratio of omega n-3 and omega n-6 oils as compared to a control where a fatty acid production antagonist is not added to the medium. Other examples can be seen in Figures 12-15.

143. The disclosed oil producing microbes, such as ONC-T18, are able to produce lipid thus obtained, any of its variants and any associations between members of the same species of eukaryotic microorganisms whereby the lipid profile is as

follows. The percentage of mono-, di- and tri-glycerides in the neutral lipid fraction of ONC-T18 is 0% to about 2%, 0 to about 2% and 96 to about 100%, respectively. While the polar lipid fraction with comprises between 5% and about 10% of the lipid fraction, comprises phosphotidylcholine, phosphotidylserine and phosphotidic acid both bound and unbound to neutral lipids.

144. It is understood that these lipids can be found in any combination or permutation within the organism. It is also understood that the concentrations of these lipids can be manipulated by changing the growing conditions and media conditions and compositions as discussed herein.

(1) Lipid as concentration

145. The disclosed oil producing microbes can produce a lipid fraction comprising n-3 DHA, EPA and n-6 DPA at greater than or equal to about 4.0 g L^{-1} of medium. The disclosed oil producing microbes can produce a lipid composition comprising n-3 DHA, EPA and n-6 DPA at greater than or equal to about 20.0 g L^{-1} of medium. The disclosed oil producing microbes can produce a lipid composition comprising n-3 DHA, EPA and n-6 DPA at greater than or equal to about 14.0 g L^{-1} of medium. The disclosed oil producing microbes can produce from about 1.5 g L^{-1} to about 5.0 g L^{-1} (e.g., about 4.6 g L^{-1}) of the n-3 DHA, from about 0.5 g L^{-1} to about 1.5 g L^{-1} (e.g., about 0.22 g L^{-1}) of the n-3 EPA, and from about 0.5 g L^{-1} to about 1.5 g L^{-1} of the n-6 DPA. Furthermore, the disclosed oil producing microbes can produce a lipid fraction comprising myristic, myristoleic, pentadecanoic, palmitic, palmitoleic, stearic oleic, linoleic, eicosadienoic, arachidonic, eicosapentaenoic, docosahexanoic and docosapentaenoic acids between 301.2 and 360.3 mg g^{-1} or even up to 790 mg g^{-1} of cellular biomass. The disclosed oil producing microbes can also produce a fraction comprising between 44.3 and 57 mg g^{-1} myristic acid (equal to 1134.5 to 1458.1 mg L^{-1}), 0.5 to 0.65 myristoleic acid (equal to 13.3 to 16.63 mg L^{-1}), 33.5 to 34.6 mg g^{-1} pentadecanoic acid (equal to 856.9 to 885.1 mg L^{-1}), 121.9 and 165.1 mg g^{-1} palmitic acid (equal to 3118.2 to 4223.3 mg L^{-1}), 7.9 to 28.5 mg g^{-1} palmitoleic acid (equal to 202.1 to 729 mg L^{-1}), 4.38 to 5.9 mg g^{-1} stearic acid (equal to 112 to 151 mg L^{-1}), 6.94 to 9.9 mg g^{-1} oleic acid (equal to 177.5 to 253.2 mg L^{-1}), 0.4 to 1.3 mg g^{-1} linoleic acid (equal to 11.26 to 33.3 mg L^{-1}), 0.5 to 1.0 mg g^{-1} eicosadienoic acid (equal to 12.8 to 25.6 mg L^{-1}), 0.4 to 0.5 mg g^{-1} arachidonic acid (equal to 10.2 to 13 mg L^{-1}), 75 to 100 mg g^{-1} docosahexanoic acid (equal to 1918 to 2560 mg L^{-1}), 1.9 to 6 mg g^{-1} eicosapenatenoic acid (equal to 48.6 to 153.5 mg L^{-1}) and 17.1 to 33.7 mg g^{-1}

docosapentaenoic acid (equal to 437.4 to 862.1 mg L^{-1}), having a total fatty acid content within the cellular biomass of between 301 to 790 mg g^{-1} (equal to 7700 to 20,209 mg L^{-1}).

146. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist sethoxydim can change the fatty acid composition for example, from 11.27% to 10.97% myristic acid, from 0.23% to 0.03% myristoleic acid, from 3.92% to 5.5% pentadecanoic acid, from 0.43% to 0.53% pentadecenoic acid, from 45.57% to 40.52% palmitic acid, from 11.63% to 9.18% palmitoleic acid, from 1.16% to 1.49% margaric acid, from 1.84% to 1.45% stearic acid, from 0.00% to 0.18% oleic acid, from 2.89% to 2.48% vaccenic acid, from 0.36% to 0.2% arachidic acid, from 0.12% to 0.00% eicosenoic acid, from 0.38% to 0.14% eicosadienoic acid, from 0.14% to 0.10% homo-gamma-linolenic acid, from 0.13% to 0.20% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.26% to 0.37% eicosatetraenoic acid, from 0.38% to 0.78% eicosapentaenoic acid, remain 0.00% behenic acid, from 1.12% to 1.09% heneicosapentaenoic acid, from 0.20% to 0.00% docosadienoic acid, from 0.16% to 0.00% adrenic acid, from 3.88% to 5.26% docosapentaenoic acid n-6, from 0.35% to 0.24% docosapentaenoic acid n-3, remain 0.00% lignoceric acid, from 13.54% to 19.31% docosahexaenoic acid, remain 0.00% nervonic acid, and from 0.27% to 0.20% hexacosanoic acid.

147. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist cerulenin can change the fatty acid composition for example, from 11.10% to 11.03% myristic acid, remain 0.00% myristoleic acid, from 3.84% to 3.23% pentadecanoic acid, from 0.11% to 0.10% pentadecenoic acid, from 50.52% to 48.98% palmitic acid, from 1.93% to 2.22% palmitoleic acid, from 0.85% to 0.74% margaric acid, from 1.55% to 1.86% stearic acid, from 0.13% to 0.29% oleic acid, from 0.93% to 1.24% vaccenic acid, from 0.38% to 0.41% arachidic acid, from 0.00% to 0.19% eicosenoic acid, remain 0.17% eicosadienoic acid, from 0.15% to 0.20% homo-gamma-linolenic acid, from 0.16% to 0.30% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.34% to 0.36% eicosatetraenoic acid, from 0.72% to 1.17% eicosapentaenoic acid, from 0.10% to 0.11% behenic acid, from 1.27% to 1.24% heneicosapentaenoic acid, from 0.13% to 0.14% docosadienoic acid, remain 0.00% adrenic acid, from 5.47% to 5.83% docosapentaenoic acid n-6, from 0.16% to 0.23% docosapentaenoic acid n-3, remain 0.00% lignoceric acid, from 19.98% to 19.97%

docosahexaenoic acid, remain 0.00% nervonic acid, and from 0.16% to 0.23% hexacosanoic acid.

148. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist oleic acid can change the fatty acid composition for example, from 24.46% to 1.38% myristic acid, from 0.03% to 0.00% myristoleic acid, from 2.43% to 0.49% pentadecanoic acid, remain 0.00% pentadecenoic acid, from 42.82% to 16.67% palmitic acid, from 0.46% to 1.06% palmitoleic acid, from 0.31% to 0.28% margaric acid, from 1.26% to 1.56% stearic acid, from 0.15% to 25.36% oleic acid, from 0.26% to 2.68% vaccenic acid, from 0.36% to 0.00% arachidic acid, remain 0.00% eicosenoic acid, from 0.06% to 0.51% eicosadienoic acid, from 0.13% to 0.61% homo-gamma-linolenic acid, from 0.22% to 0.72% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.27% to 0.00% eicosatetraenoic acid, from 0.62% to 5.47% eicosapentaenoic acid, from 0.10% to 0.00% behenic acid, from 1.01% to 0.20% heneicosapentaenoic acid, from 0.14% to 0.68% docosadienoic acid, from 0.14% to 0.60% adrenic acid, from 5.85% to 7.58% docosapentaenoic acid n-6, from 0.14% to 0.39% docosapentaenoic acid n-3, remain 0.00% lignoceric acid, from 18.77% to 33.16% docosahexaenoic acid, remain 0.00% nervonic acid, and remain 0.00% hexacosanoic acid.

149. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist oleic acid in combination with glucoase can change the fatty acid composition for example, from 24.46% to 8.04% myristic acid, from 0.03% to 0.00% myristoleic acid, from 2.43% to 0.73% pentadecanoic acid, remain 0.00% pentadecenoic acid, from 42.82% to 33.18% palmitic acid, from 0.46% to 4.22% palmitoleic acid, from 0.31% to 0.17% margaric acid, from 1.26% to 1.60% stearic acid, from 0.15% to 9.49% oleic acid, from 0.26% to 4.45% vaccenic acid, from 0.36% to 0.34% arachidic acid, from 0.00% to 0.30% eicosenoic acid, from 0.06% to 0.36% eicosadienoic acid, from 0.13% to 0.30% homo-gamma-linolenic acid, from 0.22% to 0.46% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.27% to 0.24% eicosatetraenoic acid, from 0.62% to 2.60% eicosapentaenoic acid, from 0.10% to 0.00% behenic acid, from 1.01% to 0.79% heneicosapentaenoic acid, from 0.14% to 0.43% docosadienoic acid, from 0.14% to 0.28% adrenic acid, from 5.85% to 6.46% docosapentaenoic acid n-6, from 0.14% to 0.39% docosapentaenoic acid n-3, remain 0.00% lignoceric acid, from 18.77% to 24.65% docosahexaenoic acid, remain 0.00% nervonic acid, and remain 0.00% hexacosanoic acid.

150. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist capsaicin can change the fatty acid composition for example, from 9.7% to 21.86% myristic acid, from 0.00% to 0.28% myristoleic acid, from 9.83% to 0.91% pentadecanoic acid, from 0.11% to 0.23% pentadecenoic acid, from 43.48% to 24.19% palmitic acid, from 1.52% to 17.98% palmitoleic acid, from 2.29% to 0.19% margaric acid, from 1.59% to 1.60% stearic acid, from 0.10% to 0.00% oleic acid, from 1.27% to 6.67% vaccenic acid, from 0.26% to 0.20% arachidic acid, remain 0.00% eicosenoic acid, from 0.03% to 0.23% eicosadienoic acid, from 0.25% to 0.22% homo-gamma-linolenic acid, from 0.17% to 0.78% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.30% to 0.27% eicosatetraenoic acid, from 0.48% to 1.38% eicosapentaenoic acid, from 0.08% to 0.00% behenic acid, from 0.00% to 0.00% heneicosapentaenoic acid, from 0.13% to 0.17% docosadienoic acid, from 0.07% to 0.46% adrenic acid, from 6.53% to 5.19% docosapentaenoic acid n-6, from 0.22% to 0.31% docosapentaenoic acid n-3, remain 0.00% lignoceric acid, from 21.48% to 16.86% docosahexaenoic acid, remain 0.00% nervonic acid, and remain 0.00% hexacosanoic acid.

151. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist toluic acid can change the fatty acid composition for example, from 8.95% to 11.19% myristic acid, remain 0.00% myristoleic acid, from 9.79% to 14.93% pentadecanoic acid, from 0.13% to 0.27% pentadecenoic acid, from 32.80% to 26.90% palmitic acid, from 1.94% to 2.36% palmitoleic acid, from 1.89% to 2.69% margaric acid, from 1.28% to 1.59% stearic acid, from 0.10% to 0.11% oleic acid, from 1.64% to 3.68% vaccenic acid, from 0.22% to 0.23% arachidic acid, from 0.01% to 0.14% eicosenoic acid, from 0.20% to 0.79% eicosadienoic acid, from 0.12% to 0.17% homo-gamma-linolenic acid, from 0.38% to 0.90% arachidonic acid, from 0.08% to 0.03% eicosatrienoic acid, from 0.38% to 0.36% eicosatetraenoic acid, from 1.16% to 2.95% eicosapentaenoic acid, remian 0.00% behenic acid, from 0.43% to 0.39% heneicosapentaenoic acid, from 0.09% to 0.06% docosadienoic acid, from 0.19% to 0.46% adrenic acid, from 9.43% to 5.95% docosapentaenoic acid n-6, from 0.27% to 0.54% docosapentaenoic acid n-3, from 0.07% to 0.03% lignoceric acid, from 28.48% to 23.03% docosahexaenoic acid, from 0.03% to 0.18% nervonic acid, and remain 0.00% hexacosanoic acid.

152. Lipid as percentage of total fatty acid: The presence of fatty acid production antagonist norflurazon can change the fatty acid composition for example,

from 12.36% to 11.08% myristic acid, remain 0.12% myristoleic acid, from 2.71% to 3.16% pentadecanoic acid, from 0.35% to 0.33% pentadecenoic acid, from 43.24% to 40.61% palmitic acid, from 15.54% to 10.65% palmitoleic acid, from 0.80% to 1.03% margaric acid, from 1.61% to 1.52% stearic acid, from 0.04% to 0.00% oleic acid, from 3.22% to 3.53% vaccenic acid, from 0.23% to 0.16% arachidic acid, from 0.00% to 0.02% eicosenoic acid, from 0.15% to 0.13% eicosadienoic acid, from 0.11% to 0.18% homo-gamma-linolenic acid, from 0.14% to 0.19% arachidonic acid, remain 0.00% eicosatrienoic acid, from 0.13% to 0.24% eicosatetraenoic acid, from 0.52% to 0.76% eicosapentaenoic acid, from 0.07% to 0.05% behenic acid, remain 0.00% heneicosapentaenoic acid, from 0.11% to 0.03% docosadienoic acid, from 0.23% to 0.17% adrenic acid, from 3.98% to 5.23% docosapentaenoic acid n-6, from 0.13% to 0.19% docosapentaenoic acid n-3, from 0.09% to 0.00% lignoceric acid, from 14.13% to 20.61% docosahexaenoic acid, remain 0.00% nervonic acid, and remain 0.00% hexacosanoic acid.

(2) Other molecules

153. The disclosed oil producing microbes can further produce carotenoids and xanthophylls. Examples of such carotenoids and xanthophylls include beta-carotene, lycopene, astaxanthin, canthaxanthin, phoenicoxanthin, zeaxanthin, echinenone, beta-cryptoxanthin, capsanthin, lutin, annatto, beta-apo-8-carotenal and beta-apo-8-carotenal-ester.

154. The xanthophylls produced by the disclosed oil producing microbes can be conjugated with the various PUFAs also produced by the disclosed oil producing microbes.

(a) Antioxidants

155. Generally, antioxidants are compounds that react with, and typically get consumed by oxygen. Since antioxidants typically react with oxygen, antioxidants also typically react with the free radical generators, and free radicals. ("The Antioxidants - The Nutrients that Guard Your Body" by Richard A. Passwater, Ph. D., 1985, Keats Publishing Inc., which is herein incorporated by reference at least for material related to antioxidants). The compositions can contain any antioxidants, and a non-limiting list would included but not be limited to, non-flavonoid antioxidants and nutrients that can directly scavenge free radicals including multi-carotenes, beta-carotenes, alpha-carotenes, gamma-carotenes, lycopene, lutein and zeaxanthins, selenium, Vitamin E, including alpha-, beta- and gamma- (tocopherol, particularly

alpha-tocopherol, etc., vitamin E succinate, and trolox (a soluble Vitamin E analog) Vitamin C (ascorbic acid) and Niacin (Vitamin B_3, nicotinic acid and nicotinamide), Vitamin A, 13-cis retinoic acid, , N-acetyl-L-cysteine (NAC), sodium ascorbate, pyrrolidin-edithio-carbamate, and coenzyme Q_{10}; enzymes which catalyze the destruction of free radicals including peroxidases such as glutathione peroxidase (GSHPX) which acts on H_2O_2 and such as organic peroxides, including catalase (CAT) which acts on H_2O_2, superoxide dismutase (SOD) which disproportionates $O_2H_2O_2$, glutathione transferase (GSHTx), glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PD), and mimetics, analogs and polymers thereof (analogs and polymers of antioxidant enzymes, such as SOD, are described in, for example, U.S. Patent Ser. No. 5,171,680 which is incorporated herein by reference for material at least related to antioxidants and antioxidant enzymes); glutathione; ceruloplasmin; cysteine, and cysteamine (beta-mercaptoethylamine) and flavenoids and flavenoid like molecules like folic acid and folate. A review of antioxidant enzymes and mimetics thereof and antioxidant nutrients can be found in Kumar et al, *Pharmac. Ther.* 39: 301, 1988 and Machlin L. J. and Bendich, *FASEB Journal* 1:441-445, 1987 which are incorporated herein by reference for material related to antioxidants.

156. Flavonoids, also known as "phenylchromones," are naturally occurring, water-soluble compounds which have antioxidant characteristics. Flavonoids are widely distributed in vascular plants and are found in numerous vegetables, fruits and beverages such as tea and wine (particularly red wine). Flavonoids are conjugated aromatic compounds. The most widely occurring flavonoids are flavones and flavonols (for example, myricetin, (3,5,7,3',4',5',-hexahydroxyflavone), quercetin (3,5,7,3',4'-pentahydroxyflavone), kaempferol (3,5,7,4'-tetrahydroxyflavone), and flavones apigenin (5,7,4'-trihydroxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone) and glycosides thereof and quercetin).

157. Carotenoids are important natural pigments produced by many microorganisms and plants, usually red, orange or yellow in color. Traditionally, carotenoids have been used in the feed, food and nutraceutical industries. They are known to be essential for plant growth and photosynthesis, and are a main dietary source of vitamin A in humans. Dietary antioxidants, such as carotenoids (beta-carotene, lycopene, astaxanthin, canthaxanthin, zeaxanthin, capsanthin, lutein, annatto, beta-apo-8-carotenal and beta-apo-8-carotenal-ester), exhibit significant anti-cancer

activities and play an important role in the prevention of chronic diseases. Carotenoids are potent biological antioxidants that can absorb the excited energy of singlet oxygen onto the carotenoid chain, leading to the degradation of the carotenoid molecule but preventing other molecules or tissues from being damaged.

158. Oxygen is required for metabolic functions, but it also presents challenges to cells. The human organism has a wide range of metabolic enzymes and antioxidants to rid its cells of oxygen derived molecules. This oxidative stress is supposed to be a contributing factor in conditions such as rheumatoid arthritis, ischemic heart disease and stroke, Alzheimer's dementia, cancer and ageing. Therefore, antioxidants have the potential to protect against a wide spectrum of diseases. Several antioxidant compounds have been isolated from marine microbial sources; these include astaxanthin, beta-carotene and other carotenoids.

159. Carotenoids are a widely distributed group of naturally occurring pigments, with over 700 natural lipid-soluble pigments primarily produced by microalgal, macroalgal, bacterial and fungal species, with astaxanthin and its derivatives being of particular interest commercially. Astaxanthin is an extremely effective antioxidant protector. Yet, unlike beta-carotene, astaxanthin readily crosses the blood-brain/retina barrier, and therefore also has potential to protect from diseases of the brain and the eyes. Preclinical studies suggest various beneficial effects of consuming astaxanthin such as: (i) inhibit cancer formation and growth in the bladder, colon, liver, mammary and the oral cavity; (ii) protect the retina of the eye from oxidative damage and thus has an effect against age related macular disease; (iii) promote increased immune activity, (iv) provide protection from ultraviolet light damage, as well as (v) provide increased muscle endurance.

b) Isolation of microorganisms

160. Disclosed are oil producing microbes from the family Thraustochytriaceae obtained by a method comprising baiting a vegetative sample in salt water (natural or artificial) with pollen grains and incubating; separating and transferring the grains to a heterotrophic medium and incubating; identifying an isolate that produces fatty acids, isolating from the identified isolate the microorganism from the family Thraustochytriaceae. Additional forms of isolation include media supplemented with appropriate antibiotics and identification via either by microscopic means as mentioned above or via the use of 18S rRNA gene primers or probes. The heterotrophic medium can be as described below.

5. Lipids and other molecules produced by the eukaryotic microorganism

161. Disclosed are lipid compositions comprising from about 25 wt.% to about 40 wt.% of n-3 DHA, from about 6 wt.% to about 10 wt.% of n-6 DPA, and from about 0 wt.% to about 10 wt.% of n-3 EPA.

162. The lipid composition can further comprise from about 11 wt.% to about 15 wt.% (e.g., about 13 wt.%) myristic acid, from about 7 wt.% to about 11 wt .% (e.g., about 9 wt.%) pentadecanoic acid, from about 37 wt.% to about 41 wt.% (e.g., about 39 wt.%) palmitic acid, from about 3 wt.% to about 7 wt.% (e.g., about 5 wt.%) palmitoleic acid, from about 0 to about 3 wt.% (e.g., about 1 wt.%) stearic acid, or from about 1 wt.% to about 4 wt.% (e.g., about 2 wt.%) oleic acid.

163. The lipid composition can comprise n-3 DHA in concentrations in excess of about 400 mg of biomass, n-6 DPA in concentrations in excess of 100 mg of biomass.

164. The lipid composition can further comprise carotenoids. Examples of such carotenoids include beta-carotene, lycopene, astaxanthin, zeaxanthin, canthaxanthin, echinenone, phoenicoxanthin, capsanthin, lutein, annatto, beta-apo-8-carotenal, and beta-apo-8-carotenal-ester.

165. In one aspect, the composition can comprise at least about 24 wt.% n-3 DHA, about 1 wt. % n-3 DPA, about 6 wt.% n-6 DPA, and about 1 wt.% n-3 EPA.

7. Compositions containing the molecules produced by the eukaryotic microorganism

166. A foodstuff, supplement, pharmaceutical composition for both human and animal (including marine) can comprise the composition (lipid, lipid with antioxidant and antioxidant alone).

167. Also disclosed is an infant formula comprising the composition (lipid, lipid with antioxidant and antioxidant alone).

C. Methods

1. Methods of making lipids

168. Disclosed are methods of preparing a lipid composition, the method comprising: culturing a oil producing microbecomprising one or more microorganisms from the family Thraustochytriaceae, and isolating the lipid composition.

169. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

170. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, further comprising isolating the lipid composition.

171. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is increased.

172. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is altered as compared to a contol as shown in Figure 11.

173. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is increased, wherein the overall fatty acid content is unaltered

174. Also disclosed are methods by which the fatty acid profile of said organism may be modified to increase for example EPA concentration via the supplementation of fermentation medium or such chemical compositions used in the fermentation and production of omega-3 and omega-6 fatty acids, with nutritional supplements, chemical stimuli or inhibitors which affect directly the metabolic pathway of ONC-T18. These enzymes being specifically describes as having elongase, desaturase or fatty acid synthase activities. To the inventors knowledgedate this is the first instance in which chemical compounds have been specifically identified which when combined (either throughout fermentation or at specific times points) with the fermentation medium, modify the fatty acid profile. Examples of such compounds include fatty acid intermediates present within the lipid biosynthesis pathway of this particular microorganisms, such as oleic acid (See Figure 14), which when added to the fermentation medium resultsed in an elevation of the EPA content within the cells.

175. Contrary to the experimental data of Metz et al. (2001), in which the incorporation of oleic acid into a *Schizochytrium* sp. was documented, no conversion of this product into polyunsaturated fatty acid was found to occur. In this case, through the use of ^{13}C labeled oleic acid, ^{13}C carbon originating in the enriched oleic acid was found in subsequent polyunsaturated fatty acids and constituted the

remainder of the elevated EPA concentration. This demonstrates that incorporation of intermediate fatty acids into the culture medium not only results in successful incorporation into the cells but also successful incorporation into the active desaturase, elongase and fatty acid synthase lipid synthesis system.

176. A variety of procedures can be employed in the recovery of the resultant cellular biomass from fermentation in various culture media, such as by filtration or centrifugation. The cells can then be washed, frozen, lyophilized, or spray dried, and stored under a non-oxidizing atmosphere to eliminate the presence of oxygen, prior to incorporation into a processed food or feed product.

177. Cellular lipids containing the (n-3) DHA, EPA and (n-6) DPA PUFAs can also be extracted from the cellular biomass methods such as supercritical fluid extraction, or by extraction with solvents such as chloroform, hexane, methylene chloride, or methanol, and the resulting extract evaporated under negative pressure to produce a sample of concentrated lipid material. The omega-3 and omega-6 PUFAs may be further concentrated by hydrolyzing the lipids and concentrating the highly unsaturated fraction by employing traditional methods such as urea adduction or fractional distillation, column chromatography, or by supercritical fluid fractionation. The cells can also be broken or lysed and the lipids extracted into vegetable or animal (e.g. fish oils) oils. The extracted oils can be refined by well-known processes routinely employed to refine vegetable oils (e.g. by chemical or physical refining). These refining processes remove impurities from extracted oils before they are used or sold as edible oils. After refining, the oils can be used directly as a feed or food additive to produce omega-3 and/or omega-6 enriched products. Alternatively, the oil can be further processed and purified as outlined below and then used in the above applications and also in pharmaceutical applications.

178. In another process for the production of enriched (concentrated) omega-3 or omega-6 oils, the harvested cellular biomass (fresh or dried) can be ruptured or permeabilized by well-known techniques such as sonication, liquid-shear disruption methods, bead milling, pressing under high pressure, freeze-thawing, or enzymatic digestion of the cell wall. The lipids from the ruptured cells are extracted by use of a solvent or mixture of solvents such as hexane, chloroform, ether, or methanol. The solvent is removed and the lipids hydrolyzed by using any of the well-known methods for converting triglycerides to free fatty acids or esters of fatty acids including base, acid, or enzymatic hydrolysis. After hydrolysis is completed, the nonsaponifiable

compounds are extracted into a solvent such as ether, hexane or chloroform and removed. The remaining solution is then acidified by addition of an acid, and the free fatty acid extracted into a solvent such as hexane, ether or chloroform. The solvent solution containing the free fatty acids can then be cooled to a temperature low enough for crystallization of the non-PUFA compounds, which can then be removed via filtration, centrifugation or settling. Resulting in the concentration of the remaining PUFA compounds and used as a nutritional supplements for humans, as a food additive, or as pharmaceutical applications.

179. Also, disclosed is a lipid composition prepared by the methods disclosed above.

180. The microorganisms from the family Thraustochytriaceae can be any of the microorganisms disclosed above.

a) Medium

181. The heterotrophic medium can comprise sea salt (artificial or natural), one or more carbon sources, and one or more nitrogen sources. The sea salt can be present in an amount of from about 2.0 to about 40.0 g L^{-1}. The concentration of the carbon and nitrogen source used under standard cultivation conditions (not for high-concentration, but rather cost efficient fermentation) falls within the range of 5 g L^{-1} to 60 g L^{-1} and 4 g L^{-1} to 10 g L^{-1}, respectively. For high-concentration fermentation, the concentration of the carbon and nitrogen source used under standard cultivation conditions falls within the range of 100 g L^{-1} and 160 g L^{-1} and 40 g L^{-1} to 60 g L^{-1}, respectively. The trend being that for oil accumulation, the oil producing microbe is grown in a culture medium (as those described above) in which the supply of nitrogen is limited after about 24 to about 48 hours, while the supply of carbon remains in abundance. This oil producing microbe continues to assimilate the carbon (in the form of simple sugars) but can no longer undergo cell division due to a lack of nitrogen for the generation of relevant proteins and nucleic acids. The result being that these sugars are converted into storage oils, much in the same way that Ratledge C. (*Lipid Tech.* 16:34-39, 2004) describes and figure 1 depicts this phenomenon for these organisms.

182. The nitrogen source can be one or more of peptone, yeast extract, malt extract, and sodium glutamate. The nitrogen source can also be corn steep liquor or cotton seed extract. The nitrogen source can comprise yeast extract and/or peptone or monosodium glutamate. For example, the nitrogen source can include, but is not

limited to EMD™ YE-MSG, EMD™ YE, EMD™ Peptone-MSG, Sigma™ YE-MSG, Sigma™ YE, Fermtech™ YE-MSG, Fermtech™ YE, or Fish meal (62% protein). The yeast extract can be present in an amount of about 2 g L^{-1}. The monosodium glutamate can be present in an amount of about 8 g L^{-1}.

183. The carbon source can be one or more of D-trehalose, glycerol, D-gluconic acid, L-lactic acid, D,L-malic acid, D-ribose, Tween 20, D-fructose, acetate, acetic acid, alpha-D-glucose, maltose, thymidine, L-asparagine, D-xylose, Tween 40, α-keto-glutaric acid, sucrose, L-glutamine, Tween 80, beta-methyl-D-glucoside, maltotriose, adenosinine, fumaric acid, bromo succinic acid, L-serine, D-cellobiose, L-alanyl-glycine, methyl pyruvate, L-malic acid, glycyl-L-proline, D-palcose, L-lyxose, pyruvic acid, alpha-D-lactose, dextrin, D-arabinose, 2-deoxy-D-ribose, gelatin, dextrose, starch, 3-0-beta-D-galactopyranosyl-D-arabinose, D-tagatose, 5-keto-D-gluconic acid, oxalomalic acid, sorbic acid, L-omithine, and dihydroxy acetate. In one aspect, the carbon source can be D,L-malic acid, D-fructose, D-xylose, fumaric acid, D-cellobiose, 5-keto-D-gluconic acid, pyruvic acid, alpha-D-lactose, corn dextrin, gelatin, corn starch or wheat starch.. The carbon source can be present in an amount of from about 1 g L^{-1} to about 60 g L^{-1} and up to about 200 g L^{-1}.

184. In one example, the medium can comprise about 5 g D-glucose, about 2 g peptone, and about 2 g yeast extract per liter of salt water (natural or artificial). In another, the medium can comprise about 60 g D-glucose, about 10 g yeast extract per liter of salt water (natural or artificial). In another, the medium can comprise about 8 g yeast extract, 32 g MSG, 24 g sea salt (natural and artificial) and 300 g D-glucose per liter.

185. The medium can further comprise phosphates (e.g., potassium phosphate and sodium phosphates). The medium can further comprise inorganic salts (e.g., ammonium sulfate, sodium bicarbonate, sodium orthovanadate, potassium chromate, sodium molybdate, selenous acid, nickel sulfate, copper sulfate, zinc sulfate, cobalt chloride, iron chloride, manganese chloride). The medium can further comprise a chelating compound (e.g., EDTA). The medium can further comprise vitamins (e.g., pyridoxine hydrochloride, thiamin hydrochloride, calcium pantothenate, p-aminobenzoic acid, riboflavin, nicotinic acid, biotin, folic acid, and vitamin B_{12}). The medium can be at a pH of from about 4.0 to about 6.5.

186. Incubation can be from about 1 to about 9 days (e.g., from about 3 to about 5 days). Incubation can be at from about 18 to about 30°C (e.g., from about 18-

25°C). Figure 8 shows the difference in biomass production, TFA and DHA content of the cells and glucose uptake rate when ONC-T18 is grown in fermenter under a fed-batch regime for 88 hours at both 18 and 25°C. In this instance the glucose (or carbon metabolism) to TFA production ration changes from 8.1 to 3.0. Meaning that at 18°C 8.1 g of glucose is need to generate 1 g of TFA, while at 25°C only 3.0 g of glucose is need. Incubation can further comprise shaking or aeration.

187. Isolating the lipid can comprise contacting the microorganisms with an extraction solvent. The solvent can comprise one or more solvents chosen from chloroform, hexane, methanol, or ethanol, or supercritical CO_2.

188. The method can produce any of the compositions as disclosed above.

189. The oil producing microbe can produce a lipid composition comprising n-3 DHA at greater than or equal to about 20 g L^{-1} of medium. The oil producing microbe can produce a lipid composition comprising n-3 DHA at greater than or equal to about 40 g L^{-1} of medium. The oil producing microbe can produce a lipid composition comprising n-3 DHA at greater than or equal to about 80 g L^{-1} of medium.

2. Screening and identification methods

190. The oil producing microbe as disclosed herein can produce a lipid containing the (n-3) series of docosahexaenoic acid and eicosapenatenoic acid and the (n-6) series of DPA. These oil producing microbe can be selected, for example, with the following screening method. Vegetative samples can be (and were) placed in 20 mL vials containing 10 mL of sterile 0.2 μm filtered natural seawater containing penicillin and streptomycin at 300 and 500 mg L^{-1}, respectively. The vials were then baited with sterile pollen grains and incubated for 48 hours at 18 to 25°C. The pollen grains were then transferred onto agar plates containing antibiotics (as above) and incubated under the same conditions. Single, irregular, hyaline colonies made up of spherical or limaciform cells and atypical of either yeast or bacterial colonies were picked and sub-cultured on the same medium and under the same conditions as above. These isolates were then screened for growth and fatty acids using a nutrient liquid medium, prepared with 0.2 μm filtered natural seawater containing 5 g L^{-1} glucose, 2 g L^{-1} peptone and 2 g L^{-1} yeast extract, with the resulting cellular biomass collected by centrifugation or sedimentation within a liquid medium. Fatty acids were directly transesterified using conventional methods, with the fatty acid methyl ester

composition analyzed via gas chromatography, with strains that produce appropriate amounts of the n-3 series of DHA and the n-6 series of DPA selected for further work.

191. Disclosed are methods of identifying an oil producing microbe, the method comprising: baiting a vegetative sample in salt water (natural sea or artificial) with pollen grains and incubating; transferring the grains to a heterotrophic medium and incubating; and identifying isolates that produce fatty acids.

192. Also disclosed are lipid compositions produced by the above identified oil producing microbes.

193. Also disclosed are lipid compositions produced by methods using the disclosed oil producing microbes and the methods disclosed herein.

194. Also disclosed are oil producing microbes having American Type Culture Collection accession number PTA-6245.

195. Also disclosed are oil producing microbes belonging to the order Thraustochytriales (ONC-T18) having 18S rRNA, such as SEQ ID 1, and identified as a *Thraustochytrium* sp.

196. Also disclosed are oil producing microbes belonging to the order Thraustochytriales (ONC-T18) having 18S rRNA sequence, such as GenBank accession number DQ374149, and identified as a *Thraustochytrium* sp.

197. Also disclosed is an oil producing microbe, *Thraustochytrium* sp. capable of producing DHA and DPA in concentrations in excess of 400 mg L^{-1} and 100 mg L^{-1}, respectively.

198. Also disclosed is an oil producing microbe, *Thraustochytrium* sp. capable of producing carotenoids via heterotrophic fermentation as mentioned above in the range 50 to 1250 mg kg^{-1} and astaxanthin, zeaxanthin, canthaxanthin, echinenine and beta-carotene in the range of 1 to 20 mg kg^{-1}, 0.25 to 10 mg kg^{-1}, 1 to 20 mg kg^{-1} 1 to 20 mg kg^{-1} and 1 to 200 mg kg^{-1}, respectively.

199. Also disclosed are processes for growing a oil producing microbe comprising, culturing the oil producing microbe under conditions, wherein the conditions comprise a medium comprising sodium chloride in the form of artificial sea salt (trophic marine) between 2.0 and 15.0 g L^{-1}; a nitrogen source in the form of yeast extract and monosodium glutamate at 2.0 and 8.0 g L^{-1} respectively; and carbon in the form of glucose up to 130 g L^{-1}. Also disclosed are processes for growing a oil producing microbe comprising, culturing the oil producing microbe under conditions, wherein the conditions comprise a medium comprising sodium chloride in the form of

artificial sea salt (trophic marine) between 2.0 and 15.0 g L^{-1}; a nitrogen source in the form of yeast extract and monosodium glutamate at 2.0 and 8.0 g L^{-1} respectively; and carbon in the form of glucose up to 130 g L^{-1} further comprising one or more fatty acid production antagonists.

200. The disclosed processes can, for example, grow ONC-T18, whereby at least 24% weight is DHA, at least 6% by weight is DPA and at least 1 % is EPA of total fatty acid.

201. The disclosed processes for growth can also, for example, grow ONC-T18 such that at least 1% by weight is carotenoid material, with from 1 to 2% and at least 1.2% of that being astaxanthin, with from 0.25 and 1% and at least 0.45% being zeaxanthin, with from 5 to 16% and at least 9% being canthaxanthin, with from 1 to 2% and at least 1.2% of that being echinenone and from 12 to 16% and at least 14% by weight being beta-carotene.

3. Nucleic acids

202. There are a variety of molecules disclosed herein that are nucleic acid based, including for example the nucleic acids that encode, for example, rRNA, as well as any other proteins disclosed herein, as well as various functional nucleic acids. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U/T. Likewise, it is understood that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantagous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

a) Nucleotides and related molecules

203. A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymin-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a nucleotide is pentavalent phosphate. An non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

204. A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to nucleotides are well known in the art and would include for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, and 2-aminoadenine as well as modifications at the sugar or phosphate moieties.

205. Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

206. It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid moieties such as a cholesterol moiety. (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 86:6553-6556, 1989),

207. A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

208. A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH2 or O) at the C6 position of purine nucleotides.

b) Sequences

209. There are a variety of sequences related to, for example, SEQ ID NO:1, as well as any other nucleic acids and proteins disclosed herein that can be disclosed on Genbank, and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein.

210. A variety of sequences are provided herein and these and others can be found in Genbank, at www.pubmed.gov. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and

methods relating to a particular sequence to other related sequences. Primers and/or probes can be designed for any sequence given the information disclosed herein and known in the art.

c) Primers and probes

211. Disclosed are compositions including primers and probes, which are capable of interacting with the genes disclosed herein. In certain embodiments the primers are used to support DNA amplification reactions. Typically the primers will be capable of being extended in a sequence specific manner. Extension of a primer in a sequence specific manner includes any methods wherein the sequence and/or composition of the nucleic acid molecule to which the primer is hybridized or otherwise associated directs or influences the composition or sequence of the product produced by the extension of the primer. Extension of the primer in a sequence specific manner therefore includes, but is not limited to, PCR, DNA sequencing, DNA extension, DNA polymerization, RNA transcription, or reverse transcription. Techniques and conditions that amplify the primer in a sequence specific manner are preferred. In certain aspects the primers may be used as species or genus specific probes for the *Thraustochytrium* mentioned here. In this instance, primers would be designed to be specific to the eukaryotic microorganism, with PCR reactions subsequently carried out. Presence of target species would then be determined by successful PCR product formation. In certain aspects the primers can also be used for DNA amplification reactions, such as PCR or direct sequencing. It is understood that in certain aspects the primers can also be extended using non-enzymatic techniques, where for example, the nucleotides or oligonucleotides used to extend the primer are modified such that they will chemically react to extend the primer in a sequence specific manner. Typically the disclosed primers hybridize with the nucleic acid or region of the nucleic acid or they hybridize with the complement of the nucleic acid or complement of a region of the nucleic acid.

d) Nucleic Acid Delivery

212. In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the disclosed nucleic acids can be in the form of naked DNA or RNA, or the nucleic acids can be in a vector for delivering the nucleic acids to the cells, whereby the antibody-encoding DNA fragment is under the transcriptional regulation of a promoter, as would be well understood by one of ordinary skill in the art. The

vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the disclosed nucleic acid or vector can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

213. As one example, vector delivery can be via a viral system, such as a retroviral vector system which can package a recombinant retroviral genome (see e.g., Pastan et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:4486, 1988; Miller et al., *Mol. Cell. Biol.* 6:2895, 1986). The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells nucleic acid encoding a broadly neutralizing antibody (or active fragment thereof). The exact method of introducing the altered nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors (Mitani et al., *Hum. Gene Ther.* 5:941-948, 1994), adeno-associated viral (AAV) vectors (Goodman et al., *Blood* 84:1492-1500, 1994), lentiviral vectors (Naidini et al., *Science* 272:263-267, 1996), pseudotyped retroviral vectors (Agrawal et al., *Exper. Hematol.* 24:738-747, 1996). Physical transduction techniques can also be used, such as liposome delivery and receptor-mediated and other endocytosis mechanisms (see, for example, Schwartzenberger et al., *Blood* 87:472-478, 1996). This disclosed compositions and methods can be used in conjunction with any of these or other commonly used gene transfer methods.

4. Expression systems

214. The nucleic acids that are delivered to cells typically contain expression controlling systems. For example, the inserted genes in viral and retroviral systems usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A

promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements.

215. It is understood that there are a variety of transcription control systems that can be used in the organisms disclosed herein, in addition to the general systems discussed below. It is understood that the organisms disclosed herein can be transfected and transformed with a variety of genes, such as marker genes, as discussed herein, or genes which have other desirable attributes, such as enhanced or unique growth characteristics.

a) Viral Promoters and Enhancers

216. Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature*, 273:113, 1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hin*dIII E restriction fragment (Greenway, P.J. et al., *Gene* 18:355-360, 1982). Of course, promoters from the host cell or related species also are useful herein.

217. Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., *Proc. Natl. Acad. Sci.* 78:993, 1981) or 3' (Lusky, M.L., et al., *Mol. Cell Bio.* 3:1108, 1983) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., *Cell* 33:729, 1983) as well as within the coding sequence itself (Osborne, T.F., et al., *Mol. Cell Bio.* 4:1293, 1984). They are usually between 10 and 300 bp in length, and they function in *cis*. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin (100-270 bp), the

cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

218. The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

219. In certain embodiments the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In certain constructs the promoter and/or enhancer region be active in all eukaryotic cell types, even if it is only expressed in a particular type of cell at a particular time. A preferred promoter of this type is the CMV promoter (650 bp). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

220. It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

221. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

b) Markers

222. The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Preferred marker genes are the *E. coli* *lacZ* gene, which encodes β-galactosidase, and green fluorescent protein.

223. In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR-cells and mouse LTK-cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

224. The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., *J. Molec. Appl. Genet.* 1:327, 1982), mycophenolic acid, (Mulligan, R.C. and Berg, P. *Science* 209:1422, 1980) or hygromycin, (Sugden, B. et al., *Mol. Cell. Biol.* 5:410-413, 1985). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

5. Peptides

a) Protein variants

225. As discussed herein there are numerous variants of the disclosed organism proteins that are known and herein contemplated. In addition, to the known functional Thraustochytriales strain there are derivatives of the Thraustochytriales proteins which also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such

substitutions generally are made in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

226. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

TABLE 1:Amino Acid Abbreviations

Amino Acid	Abbreviations	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

TABLE 2:Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions*
Ala	Ser
Arg	Lys; Gln
Asn	Gln; His
Asp	Glu
Cys	Ser

Gln	Asn; Lys
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln
Met	Leu; Ile
Phe	Met; Leu; Tyr
Pro	Gly
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

* Others are known in the art

227. For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

228. Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

229. Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco pp 79-86, 1983), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

230. It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. Specifically disclosed are variants of proteins herein disclosed which have at least, 60%, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

231. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482, 1981, by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443, 1970, by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

232. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

233. It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

234. As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. It is also understood that while no amino acid sequence

indicates what particular DNA sequence encodes that protein within an organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein in the particular strain from which that protein arises is also known and herein disclosed and described.

235. It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino acids or amino acids which have a different functional substituent then the amino acids shown in Table 1 and Table 2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., *Methods in Mol. Biol.* 77:43-73, 1991, Zoller, *Curr. Opin. Biotech.*, 3:348-354, 1992; Ibba, *Biotechnol. Genet. Eng.* 13:197-216, 1995, Cahill et al., *Trends Biochem. Sci.*, 14:400-403, 1989; Benner, *Trends. Biotechnol.*, 12:158-163, 1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

236. Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include CH_2NH--, --CH_2S--, --CH_2--CH_2 --, --CH=CH-- (cis and trans), --$COCH_2$ --, --$CH(OH)CH_2$--, and --CHH_2SO—(These and others can be found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267, 1983; Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, *Trends Pharm. Sci.* 463-468, 1980; Hudson, D. et al., *Int. J. Pept. Prot. Res.* 14:177-185, 1979 (--CH_2NH--, CH_2CH_2--); Spatola et al. *Life Sci.* 38:1243-1249, 1986 (--CH H_2--S); Hann J. *Chem. Soc. Perkin Trans.* I 307-314, 1982 (--CH--CH--, cis and trans); Almquist et al. *J. Med. Chem.* 23:1392-1398, 1980 (--$COCH_2$--); Jennings-White et al. *Tetrahedron Lett.*, 23:2533, 1982 (--$COCH_2$--); Szelke et al. *European Appln.*, EP 45665 CA: 97:39405, 1982 (--$CH(OH)CH_2$--); Holladay et al. *Tetrahedron Lett.* 24:4401-4404, 1983 (--$C(OH)CH_2$--); and Hruby *Life Sci.* 31:189-199, 1982 (--CH_2--S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is --CH_2NH--. It is understood that peptide

analogs can have more than one atom between the bond atoms, such as b-alanine, g-aminobutyric acid, and the like.

237. Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

238. D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch *Ann. Rev. Biochem.* 61:387, 1992, incorporated herein by reference).

6. Supplements

239. Also disclosed herein are nutritional supplements. A nutritional supplement is any compound or composition that can be administered to or taken by a subject to provide, supply, or increase a nutrient(s) (*e.g.*, vitamin, mineral, essential trace element, amino acid, peptide, nucleic acid, oligonucleotide, lipid, cholesterol, steroid, carbohydrate, and the like). In one aspect, disclosed herein are nutritional supplements comprising any of the compounds disclosed herein. For example, a nutritional supplement can comprise any of the lipids disclosed herein. The fatty acid residues of these lipids can be any fatty acid as disclosed herein (*e.g.*, unsaturated or saturated fatty acid residues).

240. The nutritional supplement can comprise any amount of the compounds disclosed herein, but will typically contain an amount determined to supply a subject with a desired dose of a benzenediol derivative (*e.g.*, CoQ_{10}) and/or fatty acids. The exact amount of compound required in the nutritional supplement will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the dietary deficiency being treated, the particular mode of administration, and the like. Thus, it is not possible to specify an exact amount for every nutritional supplement. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. In one specific example, a nutritional supplement can comprise from about

0.05 to about 20%, from about 1 to about 7.5%, or from about 3 to about 5% by weight of the compound. In another example, the nutritional supplement can comprise from about 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 10.5, 11.0, 11.5, 12.0, 12.5, 13.0, 13.5, 14.0, 14.5, 15.0, 15.5, 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5, or 20.0% by weight of the compound, where any of the stated values can form an upper or lower endpoint when appropriate. In another aspect, when the nutritional supplement, the supplement can be composed of up to 100% of the supplement.

241. The nutritional supplement can also comprise other nutrient(s) such as vitamins trace elements, minerals, and the like. Further, the nutritional supplement can comprise other components such as preservatives, antimicrobials, anti-oxidants, chelating agents, thickeners, flavorings, diluents, emulsifiers, dispersing aids, and/or binders.

242. The nutritional supplements are generally taken orally and can be in any form suitable for oral administration. For example, a nutritional supplement can typically be in a tablet, gel-cap, capsule, liquid, sachets, or syrup form.

7. Delivery Devices

243. Any of the compounds described herein can be incorporated into a delivery device. Examples of delivery devices include, but are not limited to, microcapsules, microspheres, nanospheres or nanoparticles, liposomes, noisome, nanoerythrosome, solid-liquid nanoparticles, gels, gel capsules, tablets, lotions, creams, sprays, or emulsions. Other examples of delivery devices that are suitable for non-oral administration include pulmospheres. Examples of particular delivery devices useful herein are described below.

244. The disclosed compounds can be incorporated into liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The disclosed compositions in liposome form can contain, in addition to a compound disclosed herein, stabilizers, preservatives, excipients, and the like. Examples of suitable lipids are the phospholipids and the phosphatidylcholines (lecithins), both natural and synthetic. Methods of forming liposomes are known in the art. *See, e.g.*, Prescott,

Ed., *Methods in Cell Biology*, Volume XIV, Academic Press, New York, p. 33 et seq., 1976, which is hereby incorporated by reference herein for its teachings of liposomes and their preparation.

245. In other examples, the liposomes can be cationic liposomes (*e.g.*, DOTMA, DOPE, DC cholesterol) or anionic liposomes. Liposomes can further comprise proteins to facilitate targeting a particular cell, if desired. Administration of a composition comprising a compound and a cationic liposome can be administered to the blood afferent to a target organ or inhaled into the respiratory tract to target cells of the respiratory tract. Regarding liposomes, *see e.g.*, Brigham, *et al.*, *Am J Resp Cell Mol Biol* 1:95-100, 1989; Felgner, *et al.*, *Proc Natl Acad Sci USA* 84:7413-7, 1987; and U.S. Pat. No. 4,897,355, which are incorporated by reference herein for their teachings of liposomes. As one example, delivery can be via a liposome using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. Liposomes where the diffusion of the compound or delivery of the compound from the liposome is designed for a specific rate or dosage can also be used.

246. As described herein, noisomes are delivery devices that can be used to deliver the compositions disclosed herein. Noisomes are multilamellar or unilamellar vesicles involving non-ionic surfactants. An aqueous solution of solute is enclosed by a bilayer resulting from the organization of surfactant macromolecules. Similar to liposomes, noisomes are used in targeted delivery of, for example, anticancer drugs, including methotrexate, doxorubicin, and immunoadjuvants. They are generally understood to be different from transferosomes, vesicles prepared from amphiphilic carbohydrate and amino group containing polymers, *e.g.*, chitosan.

247. As described herein, nanoerythrosomes are delivery devices that can be used to deliver the compositions disclosed herein. Nanoerythrosomes are nano-vesicles made of red blood cells via dialysis through filters of defined pore size. These vesicles can be loaded with a diverse array of biologically active molecules, including proteins and the compositions disclosed herein. They generally serve as ideal carriers for antineoplastic agents like bleomycin, actinomycin D, but can be used for steroids, other lipids, etc.

248. Artificial red blood cells, as described herein, are further delivery devices that can be used to deliver the compositions disclosed herein. Artificial red blood cells can be generated by interfacial polymerization and complex emulsion methods. Generally, the "cell" wall is made of polyphtaloyl L-lysine polymer/polystyrene and the core is made of a hemoglobin solution from sheep hemolysate. Hemoglobin loaded microspheres typically have particle sizes of from about 1 to about 10 mm. Their size, flexibility, and oxygen carrying capacity is similar to red blood cells.

249. Solid-lipid nanoparticles, as described herein, are other delivery devices that can be used to deliver the compositions disclosed herein. Solid-lipid nanoparticles are nanoparticles, which are dispersed in an aqueous surfactant solution. They are comprised of a solid hydrophobic core having a monolayer of a phospholipid coating and are usually prepared by high-pressure homogenization techniques. Immunomodulating complexes (ISCOMS) are examples of solid-lipid nanoparticles. They are cage-like 40 nm supramolecular assemblies comprising of phospholipid, cholesterol, and hydrophobic antigens and are used mostly as immunoadjuvants. For instance, ISCOMs are used to prolong blood-plasma levels of subcutaneously injected cyclosporine.

250. Microspheres and micro-capsules, as described herein, are yet other delivery devices that can be used to deliver the compositions disclosed herein. In contrast to liposomal delivery systems, microspheres and micro-capsules typically do not have an aqueous core but a solid polymer matrix or membrane. These delivery devices are obtained by controlled precipitation of polymers, chemical cross-linking of soluble polymers, and interfacial polymerization of two monomers or high-pressure homogenization techniques. The encapsulated compound is gradually released from the depot by erosion or diffusion from the particles. Successful formulations of short acting peptides, such as LHRH agonists like leuprorelin and triptoreline, have been developed. Poly(lactide co-glycolide (PLGA) microspheres are currently used as monthly and three monthly dosage forms in the treatment of advanced prostrate cancer, endometriosis, and other hormone responsive conditions. Leuprolide, an LHRH superagonist, was incorporated into a variety of PLGA matrices using a solvent extraction/evaporation method. As noted, all of these delivery devices can be used in the methods disclosed herein.

251. Pulmospheres are still other examples of delivery devices that can be used herein. Pulmospheres are hollow porous particles with a low density (less than about

0.1 m mL^{-1}). Pulmospheres typically have excellent re-dispersibility and are usually prepared by supercritical fluid condensation technology. Co-spray-drying with certain matrices, such as carbohydrates, human serum albumin, etc., can improve the stability of proteins and peptides (*e.g.*, insulin) and other biomolecules for pulmonary delivery. This type of delivery could be also accomplished with micro-emulsions and lipid emulsions, which are ultra fine, thin, transparent oil-in-water (o/w) emulsions formed spontaneously with no significant input of mechanical energy. In this technique, an emulsion can be prepared at a temperature, which must be higher than the phase inversion temperature of the system. At elevated temperature the emulsion is of water-in-oil (w/o) type and as it cools at the phase inversion temperature, this emulsion is inverted to become o/w. Due to their very small inner phase, they are extremely stable and used for sustained release of steroids and vaccines. Lipid emulsions comprise a neutral lipid core (*i.e.*, triglycerides) stabilized by a monolayer of amphiphilic lipid (*i.e.*, phospholipid) using surfactants like egg lecithin triglycerides and miglyol. They are suitable for passive and active targeting.

252. There are other oral delivery systems under investigation that are based on osmotic pressure modulation, pH modulation, swelling modulation, altered density and floating systems, mucoadhesiveness etc. These formulations and time-delayed formulations to deliver drugs in accordance with circadian rhythm of disease that are currently in use or investigation can be applied for delivery of the compositions disclosed herein.

253. In one particular aspect disclosed herein, the disclosed compounds, including nutritional supplement and pharmaceutical formulations thereof, can be incorporated into microcapsules as described herein.

254. In one aspect disclosed herein, the disclosed compounds can be incorporated into microcapsules. In one aspect, the microcapsule comprises an agglomeration of primary microcapsules and the chromium compounds described herein, each individual primary microcapsule having a primary shell, wherein the chromium compound is encapsulated by the primary shell, wherein the agglomeration is encapsulated by an outer shell. These microcapsules are referred to herein as "multicore microcapsules."

255. In another aspect, described herein are microcapsules comprising a chromium compound, a primary shell, and a secondary shell, wherein the primary shell encapsulates the chromium compound, and the secondary shell encapsulates the

loading substance and primary shell. These microcapsules are referred to herein as "single-core microcapsules.

256. Optionally, other loading substances can be encapsulated with the chromium compound. The loading substance can be any substance that is not entirely soluble in the aqueous mixture. In one aspect, the loading substance is a solid, a hydrophobic liquid, or a mixture of a solid and a hydrophobic liquid. In another aspect, the loading substance comprises a grease, an oil, a lipid, a drug (*e.g.*, small molecule), a biologically active substance, a nutritional supplement (e.g., vitamins), a flavor compound, or a mixture thereof. Examples of oils include, but are not limited to, animal oils (e.g., fish oil, marine mammal oil, etc.), vegetable oils (e.g., canola or rapeseed), mineral oils, derivatives thereof or mixtures thereof. The loading substance can be a purified or partially purified oily substance such as a fatty acid, a triglyceride or ester thereof, or a mixture thereof. In another aspect, the loading substance can be a carotenoid (e.g., lycopene), a satiety agent, a flavor compound, a drug (e.g., a water insoluble drug), a particulate, an agricultural chemical (e.g., herbicides, insecticides, fertilizers), or an aquaculture ingredient (e.g., feed, pigment).

257. In one aspect, the loading substance can be an omega-3 fatty acid. Examples of omega-3 fatty acids include, but are not limited to, α-linolenic acid (18:3n3), octadecatetraenoic acid (18:4n3), eicosapentaenoic acid (20:5n3) (EPA), docosahexaenoic acid (22:6n3) (DHA), docosapentaenoic acid (22:5n3) (DPA), eicosatetraenoic acid (20:4n3), uncosapentaenoic acid (21:5n3), docosapentaenoic acid (22:5n3) and derivatives thereof and mixtures thereof. Many types of derivatives of omega-3 fatty acids are well known in the art. Examples of suitable derivatives include, but are not limited to, esters, such as phytosterol esters, branched or unbranched C_1-C_{30} alkyl esters, branched or unbranched C_2-C_{30} alkenyl esters, or branched or unbranched C_3-C_{30} cycloalkyl esters such as phytosterol esters and C_1-C_6 alkyl esters. Sources of oils can be derived from aquatic organisms (e.g., anchovies, capelin, Atlantic cod, Atlantic herring, Atlantic mackerel, Atlantic menhaden, salmonids, sardines, shark, tuna, etc) and plants (*e.g.,* flax, vegetables, etc) and microorganisms (e.g., fungi and algae).

258. In one aspect, the loading substance can contain an antioxidant. Examples of antioxidants include, but are not limited to, vitamin E, CoQ_{10}, tocopherols, lipid soluble derivatives of more polar antioxidants such as ascorbyl fatty acid esters (e.g., ascorbyl palmitate), plant extracts (e.g., rosemary, sage and oregano oils), algal

extracts, and synthetic antioxidants (e.g., BHT, TBHQ, ethoxyquin, alkyl gallates, hydroquinones, tocotrienols).

259. A number of different polymers can be used to produce the shell layers of the single and multicore microcapsules. Examples of such polymers include, but are not limited to, a protein, a polyphosphate, a polysaccharide, or a mixture thereof. In another aspect, the shell material used to prepare the single- and multicore microcapsules further comprises In another aspect, the shell material used to prepare the single- and multicore microcapsules further comprises gelatin type A, gelatin type B, polyphosphate, gum arabic, alginate, chitosan, carrageenan, pectin, starch, modified starch, alfa-lactalbumin, beta-lactoglobumin, ovalbumin, polysorbiton, maltodextrins, cyclodextrins, cellulose, methyl cellulose, ethyl cellulose, hydropropylmethylcellulose, carboxymethylcellulose, milk protein, whey protein, soy protein, canola protein, albumin, chitin, polylactides, poly-lactide-co-glycolides, derivatized chitin, chitosan, poly-lysine, various inorganic-organic composites, or any mixture thereof. It is also contemplated that derivatives of these polymers can be used as well. In another aspect, the polymer can be kosher gelatin, non-kosher gelatin, Halal gelatin, or non-Halal gelatin.

260. In one aspect, one or more of the shell layers in the single and multicore microcapsules comprises gelatin having a Bloom number less than 50. This gelatin is referred to herein as "low Bloom gelatin." The Bloom number describes the gel strength formed at 10°C with a 6.67% solution gelled for 18 hours. In one aspect, the low Bloom gelatin has a Bloom number less than 40, less than 30, less than 20, or less than 10. In another aspect, the gelatin has a Bloom number of 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or 0, where any two values can be used to produce a range. In another aspect, the low Bloom gelatin is in both the primary shell and the outer shell of the multicore microcapsule. In one aspect, the low Bloom gelatin is gelatin type A. In another aspect, the low Bloom gelatin is gelatin type A produced by Kenney & Ross Ltd., R.R. #3 Shelburne, NS Canada. In another aspect, gelatin having a Bloom number of zero is in both the primary shell and the outer shell of the multicore microcapsule.

261. In one aspect, the material used to make the shells of the single- or multicore microcapsules is a two-component system made from a mixture of two different types of polymers. In one aspect, the material is a complex coacervate between the polymer components. Complex coacervation is caused by the interaction

between two oppositely charged polymers. In one aspect, the shell material used to produce the single and multicore microcapsules is composed of (1) low Bloom gelatin and (2) gelatin type B, polyphosphate, gum arabic, alginate, chitosan, carrageenan, pectin, carboxymethylcellulose, whey protein, soy protein, canola protein, albumin, or a mixture thereof. The molar ratio of the different polymers can vary. For example, the molar ratio of low Bloom gelatin to the other polymer component is from 1:5 to 15:1. For example, when low Bloom gelatin and polyphosphate are used, the molar ratio of low Bloom gelatin to polyphosphate is about 8:1 to about 12:1; when low Bloom gelatin and gelatin type B are used, the molar ratio is 2:1 to 1:2; and when low Bloom gelatin and alginate are used, the molar ratio is 3:1 to 8:1.

262. Processing aids can be included in the shell material (e.g., primary or outer shells). Processing aids can be used for a variety of reasons. For example, they may be used to promote agglomeration of the primary microcapsules, stabilize the emulsion system, improve the properties of the outer shells, control microcapsule size and/or to act as an antioxidant. In one aspect, the processing aid can be an emulsifier, a fatty acid, a lipid, a wax, a microbial cell (e.g., yeast cell lines), a clay, or an inorganic compound (e.g., calcium carbonate). Not wishing to be bound by theory, these processing aids can improve the barrier properties of the microcapsules. In one aspect, one or more antioxidants can be added to the shell material. Antioxidant properties are useful both during the process (*e.g.* during coacervation and/or spray drying) and in the microcapsules after they are formed (*i.e.* to extend shelf-life, etc). Preferably a small number of processing aids that perform a large number of functions can be used. In one aspect, the antioxidant can be a phenolic compound, a plant extract, or a sulphur-containing amino acid. In one aspect, ascorbic acid (or a salt thereof such as sodium or potassium ascorbate) can be used to promote agglomeration of the primary microcapsules, to control microcapsule size and to act as an antioxidant. The antioxidant can be used in an amount of about 100 ppm to about 12,000 ppm, or from about 1,000 ppm to about 5,000 ppm. Other processing aids such as, for example, metal chelators, can be used as well. For example, ethylene diamine tetraacetic acid can be used to bind metal ions, which can reduce the catalytic oxidation of the loading substance.

263. In one aspect, the primary microcapsules (primary shells) have an average diameter of about 40 nm to about 10 μm, 0.1 μm to about 10 μm, 1 μm to about 10 μm, 1 μm to about 8 μm, 1 μm to about 6 μm, 1 μm to about 4 μm, or 1 μm to about 2

μm, or 1 μm. In another aspect, the multicore microcapsules can have an average diameter of from about 1 μm to about 2000 μm, 20 μm to about 1000 μm, from about 20 μm to about 100 μm, or from about 30 μm to about 80 μm. In another aspect, the single-core microcapsules have an outer diameter of from 1 μm to 2,000 μm.

264. The microcapsules described herein generally have a combination of high payload and structural strength. For example, payloads of loading substance can be from 20% to 90%, 50% to 70% by weight, or 60% by weight of the single or multicore microcapsules.

265. In one aspect, the methods disclosed in U.S. Patent Application Publication No. 2003/0193102, which is incorporated by reference in its entirety, can be used to encapsulate the algal lipids described herein. It is also contemplated that one or more additional shell layers can be placed on the outer shell of the single or multicore microcapsules. In one aspect, the techniques described in International Publication No. WO 2004/041251 A1, which is incorporated by reference in its entirety, can be used to add additional shell layers to the single and multicore microcapsules.

a) Pharmaceutical and nutraceutical compositions

266. These lipids and antioxidants are targeted for use in animal feeds, pharmaceuticals, nutraceuticals (especially infant formula) as well as in the industry. This is to also include nutraceutical forms of delivery such as gel capsules and the like, common microencapsulations, etc.

267. As described above, the compositions can also be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

268. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant. As used herein, "topical intranasal administration" means

delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

269. Parental administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

270. The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., *Bioconjugate Chem.*, 2:447-451,1991; Bagshawe, K.D., *Br. J. Cancer*, 60:275-281,1989; Bagshawe, et al., *Br. J. Cancer*, 58:700-703,1988; Senter, et al., *Bioconjugate Chem.*, 4:3-9,1993; Battelli, et al., *Cancer Immunol. Immunother.*, 35:421-425,1992; Pietersz and McKenzie, *Immunolog. Reviews*, 129:57-80,1992; and Roffler, et al., *Biochem. Pharmacol.*, 42:2062-2065,1991). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glicoma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., *Cancer Research*, 49:6214-6220,1989); and Litzinger and Huang, *Biochimica et*

Biophysica Acta, 1104:179-187,1992). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, *DNA and Cell Biology* 10:399-409,1991).

(1) Pharmaceutically Acceptable Carriers

271. The compositions, including antibodies, can be used therapeutically in combination with a pharmaceutically acceptable carrier.

272. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

273. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

274. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

275. The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The disclosed antibodies can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

276. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

277. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

278. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

279. Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic

acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

(2) Therapeutic Uses

280. Effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are effected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example, guidance in selecting appropriate doses for antibodies can be found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noges Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 μg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

b) Targeted delivery

281. The disclosed liposomes and microcapsules can be targeted to a particular cell type, such as islets cells, via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific tissue (Senter, *et al.*, *Bioconjugate Chem* 2:447-51, 1991; Bagshawe, *Br J Cancer* 60:275-81, 1989; Bagshawe, *et al.*, *Br J Cancer* 58:700-3, 1988; Senter, *et al.*, *Bioconjugate Chem* 4:3-9, 1993; Battelli, *et al.*, *Cancer Immunol Immunother* 35:421-5, 1992; Pietersz and McKenzie, *Immunolog Reviews* 129:57-80, 1992; and Roffler, *et al.*,

Biochem Pharmacol 42:2062-5, 1991). These techniques can be used for a variety of other specific cell types.

8. Foodstuffs

282. Also disclosed herein are foodstuffs comprising any of the microcapsules and emulsions disclosed herein. By "foodstuff" is meant any article that can be consumed (e.g., eaten, drank, or ingested) by a subject. In one aspect, the microcapsules can be used as nutritional supplements to a foodstuff. For example, the microcapsules and emulsions can be loaded with vitamins, omega-3 fatty acids, and other compounds that provide health benefits. In one aspect, the foodstuff is a baked good, a pasta, a meat product, a frozen dairy product, a milk product, a cheese product, an egg product, a condiment, a soup mix, a snack food, a nut product, a plant protein product, a hard candy, a soft candy, a poultry product, a processed fruit juice, a granulated sugar (*e.g.*, white or brown), a sauce, a gravy, a syrup, a nutritional bar, a beverage, a dry beverage powder, a jam or jelly, a fish product, or pet companion food. In another aspect, the foodstuff is bread, tortillas, cereal, sausage, chicken, ice cream, yogurt, milk, salad dressing, rice bran, fruit juice, a dry beverage powder, rolls, cookies, crackers, snack food, fruit pies, or cakes.

9. Chips and microarrays

283. Disclosed are chips where at least one address is the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also disclosed are chips where at least one address is the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

284. Also disclosed are chips where at least one address is a variant of the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also disclosed are chips where at least one address is a variant of the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

10. Computer readable mediums

285. It is understood that the disclosed nucleic acids and proteins can be represented as a sequence consisting of the nucleotides of amino acids. There are a variety of ways to display these sequences, for example the nucleotide guanosine can be represented by G or g. Likewise the amino acid valine can be represented by Val or V. Those of skill in the art understand how to display and express any nucleic acid or protein sequence in any of the variety of ways that exist, each of which is considered

herein disclosed. Specifically contemplated herein is the display of these sequences on computer readable mediums, such as, commercially available floppy disks, tapes, chips, hard drives, compact disks, and video disks, or other computer readable mediums. Also disclosed are the binary code representations of the disclosed sequences. Those of skill in the art understand what computer readable mediums. Thus, computer readable mediums on which the nucleic acids or protein sequences are recorded, stored, or saved.

286. Disclosed are computer readable mediums comprising the sequences and information regarding the sequences set forth herein.

11. Kits

287. Disclosed herein are kits that are drawn to reagents that can be used in practicing the methods disclosed herein or in using or keeping the compositions disclosed herein. The kits can include any reagent or combination of reagent discussed herein or that would be understood to be required or beneficial in the practice of the disclosed methods. For example, the kits could include one or more of the eukaryotic microorganisms disclosed herein along with, for example, media for their maintenance. The kits could also include, for example, the lipids or antioxidants, along with means for using or administering these.

12. Compositions with similar functions

288. It is understood that the compositions disclosed herein have certain functions, such as producing certain ratios of lipids. Disclosed herein are certain structural, genetic, and functional requirements for performing the disclosed functions, and it is understood that there are a variety of structures, genetic backgrounds, and functional backgrounds which can perform the same function which are related to the disclosed structures, and that these structures will ultimately achieve the same result, for example production of a certain ration of lipids.

D. Methods of making the compositions

289. The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

1. Nucleic acid synthesis

290. For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from

standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al.*, *Ann. Rev. Biochem.* 53:323-356, 1984, (phosphotriester and phosphite-triester methods), and Narang *et al.*, *Methods Enzymol.*, 65:610-620, 1980, (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* 5:3-7, 1994.

2. Peptide synthesis

291. One method of producing the disclosed proteins is to link two or more peptides or polypeptides together by protein chemistry techniques. For example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert* -butyloxycarbonoyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) Synthetic Peptides: A User Guide. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) Principles of Peptide Synthesis. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

292. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide

fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151, 1991). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. *Science*, 266:776-779, 1994). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. *FEBS Lett.* 307:97-101, 1992; Clark-Lewis I et al., *J. Biol. Chem.*, 269:16075, 1994; Clark-Lewis I et al., *Biochemistry*, 30:3128, 1991; Rajarathnam K et al., *Biochemistry* 33:6623-30, 1994).

293. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221, 1992). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (de Lisle Milton RC et al., Techniques in Protein Chemistry IV. Academic Press, New York, pp. 257-267, 1992).

3. Processes for making the compositions

294. Disclosed are processes for making the compositions as well as making the intermediates leading to the compositions. For example, disclosed are eukaryotic microorganisms which can produce desired lipids and antioxidants as well as methods for isolating and purifying the desired lipids and antioxidants. There are a variety of methods that can be used for making these compositions, such as synthetic chemical methods and standard molecular biology methods. It is understood that the methods of making these and the other disclosed compositions are specifically disclosed.

295. Disclosed are cells produced by the process of transforming the cell with any nucleic acid. Disclosed are cells produced by the process of transforming the cell with any of the non-naturally occurring disclosed nucleic acids.

296. Disclosed are any of the lipids produced by the disclosed eukaryotic microorganisms. Disclosed are any peptides produced by the process of expressing the peptide in the disclosed organisms. Methods of using the compositions.

4. Methods of using the compositions as research tools

297. The disclosed compositions can be used in a variety of ways as research tools and of the production of, for example, lipids and antioxidants.

298. The disclosed compositions can be used as discussed herein as either reagents in micro arrays or as reagents to probe or analyze existing microarrays. The disclosed compositions can be used in any known method for isolating or identifying single nucleotide polymorphisms. The compositions can also be used in any method for determining allelic analysis of for example, the strains of the organisms disclosed herein, particularly allelic analysis as it relates to the production of lipids and antioxidants. The compositions can also be used in any known method of screening assays, related to chip/microarrays. The compositions can also be used in any known way of using the computer readable embodiments of the disclosed compositions, for example, to study relatedness or to perform molecular modeling analysis related to the disclosed compositions.

5. Methods of gene modification and gene disruption

299. The disclosed compositions and methods can be used for targeted gene disruption and modification in any animal that can undergo these events. Gene modification and gene disruption refer to the methods, techniques, and compositions that surround the selective removal or alteration of a gene or stretch of chromosome in an organism, such as the eukaryotes disclosed herein, in a way that propagates the modification through the replication of the organism. In general, for example, a cell is transformed with a vector which is designed to homologously recombine with a region of a particular chromosome or nucleic acid contained within the cell, as for example, described herein. This homologous recombination event can produce a chromosome which has exogenous DNA introduced, for example in frame, with the surrounding DNA. This type of protocol allows for very specific mutations, such as point mutations, to be introduced into the genome contained within the cell. Methods for performing this type of homologous recombination are disclosed herein.

V. SPECIFIC EMBODIMENTS

300. Disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

301. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, further comprising isolating the lipid composition.

302. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is increased.

303. Also disclosed are methods of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is increased, wherein the overall fatty acid content is unaltered.

304. The oil producing microbe for the methods described herein can be a diatom, a microalgae, a fungi, a bacterium, a protist. For example, the oil producing microbe has an 18S sequence, wherein the 18S sequence has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % identity to the sequence set forth in SEQ ID NO:1 or the oil producing microbe has an 18S sequence, wherein the 18S sequence has at least 80% identity to the sequence set forth in SEQ ID NO:1

305. The oil producing microbe can be from the phylum Labyrinthulomycota, the class Labyrinthulomycetes, the subclass Thraustochytridae, the order Thraustochytriales, the family Thraustochytriaceae, or from the genus Thraustochytrium.

306. Also disclosed are fatty acid production antagonists that can be used in the disclosed methods. The fatty acid production antagonists can be selected from the group consisting of oleic acid, cerulenin, toluic acid, curcumin, cyclopropene, alkyl gallate, propyl gallate, sesame oil, peanut oil, canola oil, pyridazinone, norflurazon, diphenylamine, sethoxydim, and capsaicin.

307. Also disclosed are delivery devices comprising the disclosed compositions..The delivery devices can comprise a microcapsule, a microsphere, a nanosphere or nanoparticle, a liposome, a noisome, a nanoerythrosome, a solid-liquid nanoparticle, a leuprolide, a gel, a gel capsule, a tablet, a lotion, a cream, a spray, an emulsion, or a powder.

308. Also disclosed are methods of preparing a carotenoid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

309. Also disclosed are methods of preparing a carotenoid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, further comprising isolating the carotenoid composition.

310. Also disclosed are methods of preparing an antioxidant composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

311. Also disclosed are methods of preparing an antioxidant composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist, further comprising isolating the antioxidant composition.

312. Also disclosed are microcapsules, comprising an agglomeration of primary microcapsules and a loading substance, each individual primary microcapsule having a primary shell, wherein the loading substance comprises any of the compositions described above, and is encapsulated by the primary shell, and wherein the agglomeration is encapsulated by an outer shell. The primary shell and/or outer shell can comprise a surfactant, gelatin, polyphosphate, polysaccharide, or a mixture thereof. The primary shell and/or outer shell can also comprise gelatin type B, polyphosphate, gum arabic, alginate, chitosan, carrageenan, pectin, starch, modified starch, alfa-lactalbumin, beta-lactoglobumin, ovalbumin, polysorbiton, maltodextrin, cyclodextrin, cellulose, methyl cellulose, ethyl cellulose, hydropropylmethylcellulose, carboxymethylcellulose, milk protein, whey protein, soy protein, canola protein, albumin, kosher gelatin, non-kosher gelatin, Halal gelatin, non-Halal gelatin, or a mixture thereof. The primary shell and/or outer shell can also comprise a complex coacervate, gelatin type A, fish gelatin, a gelatin with a Bloom number of from about 0 to about 300, a gelatin with a Bloom number of from about 0 to about 50, a gelatin with a Bloom number of from about 51 to about 300, a gelatin with a Bloom number of about 0, about 210, about 220, or about 240, a coacervate of gelatin and polyphosphate.

313. The loading substance of the disclosed microcapsules can comprise oil from any of the disclosed oil producing microbes, or a mixture thereof. The loading

substance can be from about 20% to about 90% or 50% to about 70% by weight of the microcapsule.

314. The outer shell of the disclosed microcapsules can have an average diameter of from about 1 μm to about 2,000 μm, about 20 μm to about 1,000 μm, about 30 μm to about 80 μm, about 40 nm to about 10 μm, or about 0.1 μm to about 5 μm.

315. Also disclosed is a nutritional supplement that comprises any of the compositions, delivery devices, or microcapsules described above. The disclosed nutritional supplements can be in the form of a tablet, gel-cap, capsule, liquid, or syrup.

316. Also disclosed are foodstuffs that comprises any of the compositions, delivery devices, or microcapsules described above. The foodstuff can be a baked good, a pasta, a meat product, a frozen dairy product, a milk product, a cheese product, an egg product, a condiment, a soup mix, a snack food, a nut product, a plant protein product, a hard candy, a soft candy, a poultry product, a processed fruit juice, a granulated sugar, a sauce, a gravy, a syrup, a nutritional bar, a beverage, a dry beverage powder, a jam or jelly, an infant formula, or a baby food. The foodstuff can also be a fish product, a companion pet food, a livestock or an aquaculture feed.

317. The foodstuff can also be bread, tortillas, cereal, sausage, chicken, ice cream, yogurt, milk, salad dressing, rice bran, fruit juice, a dry beverage powder, rolls, cookies, crackers, fruit pies, or cakes.

318. Also disclosed are methods of delivering a composition to a subject, comprising administering to the subject any of the compositions, delivery devices, microcapsules, or foodstuffs described above. The subject can be a mammal. The subject can also be a human.

319. Also disclosed is a use of any of the microcapsules described above and to prepare a medicament for delivering a loading substance to a subject.

320. Also disclosed is a method of lowering cholesterol levels, triglyceride levels, or a combination thereof in a subject, comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

321. Also disclosed is a method of supplementing essential trace elements in a subject, the method comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional

supplements, or foodstuffs described above, wherein the composition, delivery device, microcapsule, supplement, and foodstuff comprises an essential trace element.

322. Also disclosed is a method of improving insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

323. Also disclosed is a method of reducing hyperglycemia in a subject, comprising the step of administering to the subject an effective amount of of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

324. Also disclosed is a method of reducing hypercholesterolemia in a subject, comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

325. Also disclosed is a method of reducing body fat in a subject, comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

326. Also disclosed is a method of promoting weight loss in a subject, comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

327. Also disclosed is a method of treating or preventing diabetes in a subject, comprising the step of administering to the subject an effective amount of any of the compositions, delivery devices, microcapsules, nutritional supplements, or foodstuffs described above.

328. Also disclosed is a pharmaceutical formulation comprising any of the compositions, delivery devices, or microcapsules described above, and a pharmaceutical carrier.

VI. Examples

329. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure.

Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1 Isolation of ONC-T18 *Thraustochytrium* sp. strain

330. Classical bacteriological strain purification techniques were employed in order to isolate ONC-T18 from mangrove leaves collected at Advocate Harbor, Nova Scotia. ONC-T18 was serially cultured at 25°C on a nutrient medium agar containing 5 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} yeast extract and 15.0 g L^{-1} agar to 1L of 0.2 μm filtered sea water until purity was assured. Subsequently, a liquid medium containing 15% artificial sea water (trophic marine), supplemented with a nitrogen and carbon source, being 60 g L^{-1} glucose and 10 g L^{-1} yeast extract, respectively, was prepared. This medium (50 ml of medium in 250 ml flasks) was inoculated with ONC-T18, then incubated at 25°C and aerated via shaking at 120 rpm.

331. ONC-T18 was separated from medium via centrifugation, with cellular biomass then washed, recentrifuged and freeze dried to completion. Cellular biomass was then weighed in order to determine culture efficiencies with biomass per liter medium values recorded. Extraction of lipid fraction from biomass and subsequent fatty acid methyl ester separation was performed using the Bligh & Dyer method. Transesterification was performed by transferring freeze dried cellular material to a 10 ml screw-top test tube and adding 10% methanolic HCl and dichloromethane to the tube, with the mixture allowed to react for 2 hours at 90°C. Fatty acid methyl esters were then extracted via addition of hexane:chloroform, and the methyl ester component measured via gas chromatography (FID) in order to determine the fatty acid profile of each microorganism and the symbiotic community (ONC-T18).Concentrations of each fatty acid methyl ester (C14:0 to C22:6) were determined by comparison of the GC peak areas of two internal standards (C19:0 and C23:0) added in defined amounts both at the beginning (C23:0) and end (C19:0) of the transesterification process. The total amount of fatty acids per gram of dried cell biomass and the percentage content of each fatty acid, calculated using this method, are shown in figure 2.

332. From analysis of these results, in conjunction with those shown in Figure 1, it can be seen that ONC-T18 demonstrated the ability to produce increased amounts of DHA, as well as marked quantities of EPA and DPA. ONC-T18, produces of

approximately 25% DHA, 8.0 % (n-6) DPA and 1.0 % EPA within this non-optimized fermentation medium. Subsequently, ONC-T18 was chosen on the basis of a combination of economically desirable characteristics: (1) capable of maximal heterotrophic growth (compared to control strains); (2) contain a high percentage of omega-3 highly unsaturated fatty acids; (3) capable of growth on inexpensive nutrients; (4) thermotolerance, and are (5) euryhaline.

333. In addition, multiple different strains of oil producing microbes were compared to ONC-T18. Each of these microbes is believed to comprise a Thraustochyrid, and produces oil in the amounts shown in Table 3.

Table 3, [ ] = mg/g

	[ ] DHA	[ ] EPA	Total Lipid	Weight (g)
MYA-1381	127.96	5.52	216.37	1.80
ATCC-20891	37.97	7.14	67.34	1.30
ONC-T01	5.18	7.76	50.82	0.40
ONC-T02	31.84	4.20	52.88	0.50
ONC-T03	24.87	6.97	75.00	0.60
ONC-T04	14.39	4.49	41.74	0.90
ONC-T05	11.37	3.97	34.89	0.10
ONC-T06	27.80	6.71	63.87	0.10
ONC-T07	33.02	5.49	61.81	0.50
ONC-T08	24.48	4.83	53.35	0.80
ONC-T09	63.82	4.25	109.12	0.80
ONC-T10	22.22	4.93	40.99	0.10
ONC-T11	18.37	21.25	214.98	0.80
ONC-T12	57.96	9.03	96.26	0.60
ONC-T13	12.90	4.57	39.52	1.30
ONC-T14	15.99	5.16	36.46	0.50
ONC-T15	15.53	5.11	37.72	0.50
ONC-T16	18.02	5.55	42.12	0.50
ONC-T17	36.43	4.34	94.26	0.30
ONC-T18	83.63	2.76	321.14	2.30
ONC-T19	34.71	8.07	66.14	0.60
ONC-T20	19.28	6.94	66.74	0.10
ONC-T21				
ONC-T22	22.72	3.26	47.58	0.60
ONC-T23				
ONC-T24	11.73	3.56	33.56	0.70
ONC-T25	26.99	6.11	45.67	0.60
ONC-T26	14.50	6.43	39.22	0.60
ONC-T27	26.83	7.75	61.87	0.70
ONC-T28	16.62	6.02	38.28	0.90
ONC-T29	14.67	4.91	34.48	0.80
ONC-T30	16.56	5.42	81.88	0.80
ONC-T31	13.36	5.74	44.86	0.30
ONC-T32	19.12	6.56	53.29	0.20
ONC-T33				
ONC-T34	18.92	5.98	53.36	0.60
ONC-T35				
ONC-T36				

ONC-T37	35.69	11.06	82.73	0.10
ONC-T38	22.73	10.94	51.56	0.10
ONC-T39				
ONC-T40	26.87	8.83	67.87	0.80
ONC-T41	22.85	6.65	52.63	0.50
ONC-T42	33.65	9.22	83.93	0.80
ONC-T43	12.49	3.25	37.93	0.80
ONC-T44	11.71	2.93	55.05	1.10
ONC-T45	26.08	7.95	70.45	0.70
ONC-T46	33.34	6.27	63.76	0.30
ONC-T47	10.01	4.77	68.02	0.70
ONC-T48	26.23	3.95	69.06	0.60
ONC-T49	16.64	4.89	39.76	0.30
ONC-T50	13.64	4.56	40.30	1.00
ONC-T51				
ONC-T52	26.57	4.55	41.36	0.60
ONC-T53	11.40	3.56	29.20	0.70
ONC-T54	10.34	3.18	29.31	0.70
ONC-T55				
ONC-T56				
ONC-T57				
ONC-T58	10.30	3.13	27.10	0.70
ONC-T59				
ONC-T60	27.71	7.01	66.84	0.30
ONC-T61	15.72	5.62	52.56	0.40
ONC-T62				
ONC-T63	20.17	8.25	62.58	0.60
ONC-T64	12.16	2.97	44.73	1.10
ONC-T65				
ONC-T66				
ONC-T67	23.71	5.63	43.24	0.50
ONC-T68	22.72	6.10	41.37	0.50

334. It is understood that just as for ONC-T18, as described herein, a set of oil producing microbes as represented by the oil producing capablilities disclosed herein are disclosed, such as by a percentage of DHA to total oil production, or by total DHA production, for example.

2. Example 2 Identification eukaryotic *Thraustochytrium* species of ONC-T18 using genetic techniques

335. Using polymerase chain reaction (PCR) techniques and primers targeting the 18S ribosomal RNA gene, being universal for all eukaryotic species, it was possible to generate PCR products of the structural genes of the oil producing microbe isolated from ONC-T18 (as per example 1). PCR products were then sequenced and designated SEQ ID NO:1 for the eukaryotic species (see figure 2).

336. Comparison of SEQ ID NO:1 with nucleic acid sequences found in the genomic database, GenBank (National Centre for Biotechnology Information, National Institute of Health, Bethesda, MD, USA) using the BLAST (Basic local

alignment search tool) algorithm identified SEQ ID NO:1 as being most related to *Thraustochytrium striatum* [AF265338] (97.5% similarity).

337. BLAST results for ONC-T18 *Thraustochytrium* sp. are shown below.

Sequences producing significant alignments:	Score (bits)	E Value
gi|14279326|gb|AF265338.1| Thraustochytrium striatum small subun...	2126	0.0
gi|50508012|dbj|AB183657.1| Thraustochytriidae sp. MBIC11072 gen...	2121	0.0
gi|54778780|gb|AY773276.1| Thraustochytriidae sp. FJN-10 18S rib...	1857	0.0
gi|50508019|dbj|AB183664.1| Thraustochytriidae sp. MBIC11093 gen...	1828	0.0
gi|38524571|dbj|AB126669.1| Thraustochytrium sp. CHN-1 gene for ...	1748	0.0
gi|24817740|dbj|AB073308.2| Thraustochytriidae sp. N1-27 gene fo...	1628	0.0
gi|50508018|dbj|AB183663.1| Thraustochytriidae sp. MBIC11092 gen...	1257	0.0
gi|50508017|dbj|AB183662.1| Thraustochytriidae sp. MBIC11091 gen...	1257	0.0
gi|50508015|dbj|AB183660.1| Thraustochytriidae sp. MBIC11084 gen...	1255	0.0
gi|50508011|dbj|AB183656.1| Thraustochytriidae sp. MBIC11070 gen...	1255	0.0
gi|50508016|dbj|AB183661.1| Thraustochytriidae sp. MBIC11086 gen...	1249	0.0
gi|15823623|dbj|AB052555.1| Schizochytrium sp. KH105 gene for 18...	1245	0.0
gi|50508013|dbj|AB183658.1| Thraustochytriidae sp. MBIC11075 gen...	1227	0.0
gi|50508010|dbj|AB183655.1| Thraustochytriidae sp. MBIC11067 gen...	1213	0.0
gi|54303872|gb|AY758384.1| Schizochytrium sp. FJU-512 18S riboso...	1158	0.0
gi|14279326|gb|AF265338.1|AF265338 Thraustochytrium striatum sma...	1106	0.0
gi|6492308|gb|AF155209.1|AF155209 Labyrinthulid quahog parasite ...	765	0.0
gi|16209570|gb|AY052644.1| Labyrinthulid quahog parasite QPX sma...	757	0.0
gi|9755031|gb|AF261664.1|AF261664 Labyrinthulid quahog parasite ...	757	0.0
gi|58176547|gb|AY870336.1| Thraustochytriidae sp. Fng1 18S ribos...	735	0.0
gi|67624914|dbj|AB191425.1| Uncultured eukaryote gene for small ...	724	0.0
gi|5509891|dbj|AB022112.1| Thraustochytrium striatum gene for 18...	724	0.0
gi|561884|gb|L34054.1|ULKRRE Ulkenia profunda 18S ribosomal RNA ...	702	0.0
gi|50508014|dbj|AB183659.1| Thraustochytriidae sp. MBIC11077 gen...	686	0.0
gi|50508008|dbj|AB183653.1| Thraustochytriidae sp. MBIC11060 gen...	686	0.0
gi|50508009|dbj|AB183654.1| Thraustochytriidae sp. MBIC11063 gen...	658	0.0
gi|41391986|emb|AJ535188.1| Pleurosira cf. laevis 18S rRNA gene,...	634	e-178
gi|28316562|gb|AF525670.1| Pleurosira laevis small subunit ribos...	634	e-178
gi|5509889|dbj|AB022110.1| Thraustochytrium aureum gene for 18S ...	634	e-178
gi|561883|gb|L34668.1|TUKRRE Thraustochytrium kinnei 18S ribosom...	628	e-176
gi|5509894|dbj|AB022115.1| Ulkenia radiata gene for 18S rRNA	624	e-175
gi|5509893|dbj|AB022114.1| Ulkenia profunda gene for 18S rRNA	624	e-175
gi|5509895|dbj|AB022116.1| Ulkenia visurgensis gene for 18S rRNA	603	e-169
gi|9027563|gb|AF257315.2| Thraustochytriidae sp. BS2 18S ribosom...	589	e-164
gi|5509886|dbj|AB022107.1| Schizochytrium limacinum gene for 18S...	581	e-162
gi|48727879|gb|AY620254.1| Metromonas simplex clone TC-S small s...	571	e-159
gi|33309650|gb|AF411282.1| Unidentified cercozoan 18S ribosomal ...	569	e-158
gi|28076844|gb|AF530543.1| Uncultured eukaryote clone AT4-68 18S...	531	e-147
gi|30144485|gb|AY256273.1| Uncultured eukaryote isolate E170 sma...	517	e-143
gi|30144529|gb|AY256317.1| Uncultured eukaryote isolate D107 sma...	507	e-140
gi|14579477|gb|AF363207.1| Eukaryote marine clone ME1-24 18S rib...	505	e-139
gi|39578677|gb|AY426906.1| Uncultured marine eukaryote clone BL0...	504	e-139
gi|39981869|gb|AY381216.1| Uncultured eukaryote clone BL010625.3...	504	e-139

gi|73533408|gb|DQ103811.1| Uncultured marine eukaryote clone M4_... 504 e-139
gi|73533402|gb|DQ103805.1| Uncultured marine eukaryote clone M3_... 504 e-139
gi|73533389|gb|DQ103792.1| Uncultured marine eukaryote clone M2_... 504 e-139
gi|73533382|gb|DQ103785.1| Uncultured marine eukaryote clone M1_... 504 e-139
gi|30144534|gb|AY256322.1| Uncultured eukaryote isolate D179 sma... 504 e-139
gi|24817738|dbj|AB073305.2| Thraustochytriidae sp. H1-14 gene fo... 504 e-139
gi|30268157|emb|AJ519935.1|AST519935 Aplanochytrium stocchinoi p... 504 e-139
gi|58531881|gb|AY882527.1| Uncultured marine eukaryote clone T41... 504 e-139
gi|463127|gb|L27634.1|LADDLRRNA Labyrinthuloides minuta 16S-like... 504 e-139
gi|39981839|gb|AY381186.1| Uncultured eukaryote clone OR000415.1... 502 e-138
gi|39981824|gb|AY381171.1| Uncultured eukaryote clone HE001005.1... 502 e-138
gi|18026024|gb|AY046848.1| Uncultured eukaryote isolate C3_E019 ... 502 e-138
gi|18026022|gb|AY046846.1| Uncultured eukaryote isolate C3_E017 ... 502 e-138
gi|18026014|gb|AY046838.1| Uncultured eukaryote isolate C3_E008 ... 502 e-138
gi|18026008|gb|AY046832.1| Uncultured eukaryote isolate C3_E002 ... 502 e-138
gi|18025980|gb|AY046804.1| Uncultured eukaryote isolate C2_E014 ... 502 e-138
gi|18025969|gb|AY046793.1| Uncultured eukaryote isolate C2_E002 ... 502 e-138
gi|18025801|gb|AY046625.1| Uncultured eukaryote isolate C1_E024 ... 502 e-138
gi|67624915|dbj|AB191426.1| Uncultured eukaryote gene for small ... 502 e-138
gi|67624913|dbj|AB191424.1| Uncultured eukaryote gene for small ... 502 e-138
gi|67624912|dbj|AB191423.1| Uncultured eukaryote gene for small ... 502 e-138
gi|39981861|gb|AY381208.1| Uncultured eukaryote clone BL010320.1... 500 e-138
gi|14349249|dbj|AB052556.1| Thraustochytrium sp. KK17-3 gene for... 500 e-138
gi|20218962|dbj|AB073307.1| Thraustochytriidae sp. M4-103 gene f... 498 e-137
gi|59709960|gb|AY916582.1| Uncultured eukaryote clone Zeuk76 18S... 496 e-136
gi|18025960|gb|AY046784.1| Uncultured eukaryote isolate A3_E043 ... 496 e-136
gi|18025789|gb|AY046613.1| Uncultured eukaryote isolate C1_E009 ... 496 e-136
gi|30144548|gb|AY256336.1| Uncultured eukaryote isolate D278 sma... 496 e-136
gi|2138106|gb|U59933.1|U59933 Scybalium jamaicense 18S ribosomal... 496 e-136
gi|53828186|gb|AY744948.1| Phytophthora palmivora isolate 88108 ... 494 e-136
gi|60687349|gb|AY821976.1| Uncultured oomycete clone CV1_B2_5 sm... 494 e-136
gi|60687347|gb|AY821974.1| Uncultured Phytophthora-like oomycete... 494 e-136
gi|60687342|gb|AY821969.1| Uncultured oomycete clone CV1_B1_49 s... 494 e-136
gi|39981870|gb|AY381217.1| Uncultured eukaryote clone BL010625.3... 494 e-136
gi|39981864|gb|AY381211.1| Uncultured eukaryote clone BL010320.2... 494 e-136
gi|39981860|gb|AY381207.1| Uncultured eukaryote clone BL010320.6... 494 e-136
gi|39981844|gb|AY381191.1| Uncultured eukaryote clone BL000921.1... 494 e-136
gi|18026046|gb|AY046870.1| Uncultured eukaryote isolate C3_E044 ... 494 e-136
gi|18026039|gb|AY046863.1| Uncultured eukaryote isolate C3_E035 ... 494 e-136
gi|18026031|gb|AY046855.1| Uncultured eukaryote isolate C3_E026 ... 494 e-136
gi|42412527|gb|AY486144.1| Pythium insidiosum 18S ribosomal RNA ... 494 e-136
gi|73533425|gb|DQ103828.1| Uncultured marine eukaryote clone M2_... 494 e-136
gi|34576227|gb|AY129064.1| Uncultured marine eukaryote UEPAC45p4... 494 e-136
gi|30144522|gb|AY256310.1| Uncultured eukaryote isolate D85 smal... 494 e-136
gi|30144521|gb|AY256309.1| Uncultured eukaryote isolate D84 smal... 494 e-136
gi|30144518|gb|AY256306.1| Uncultured eukaryote isolate D79 smal... 494 e-136
gi|30144475|gb|AY256263.1| Uncultured eukaryote isolate E106 sma... 494 e-136
gi|30144473|gb|AY256261.1| Uncultured eukaryote isolate E94 smal... 494 e-136
gi|21954246|gb|AY116220.1| Uncultured eukaryote clone ANT12-26 1... 494 e-136
gi|41393027|emb|AJ535176.1|LMI535176 Leptocylindrus minimum 18S ... 494 e-136
gi|53693111|gb|AY742743.1| Phytophthora tropicalis isolate 129F-... 494 e-136

gi|53693108|gb|AY742759.1| Pythium vexans isolate Pyv6-2 18S rib... 494 e-136
gi|53693105|gb|AY742756.1| Pythium splendens isolate 117 18S rib... 494 e-136
gi|53693104|gb|AY742755.1| Pythium aphanidermatum 18S ribosomal ... 494 e-136
gi|53693097|gb|AY742748.1| Phytophthora capsici isolate 98110 18... 494 e-136
gi|53693096|gb|AY742747.1| Phytophthora tropicalis isolate 23047... 494 e-136
gi|53693094|gb|AY742745.1| Phytophthora palmivora isolate 8829 1... 494 e-136
gi|58531862|gb|AY882508.1| Uncultured marine eukaryote clone T53... 494 e-136

3. Example 3: Optimized production of biomass using strain ONC-T18

338. Production of microbial derived (or single celled) oils are dependent on a variety of process variables, such as initial inoculum level/concentration, type of substrate, media composition, temperature and pH. Specifically, microbial-based production of highly unsaturated fatty acids using *Thraustochytrid* strains, shows a direct correlation between biomass and fatty acid production. Consequently, an understanding of the basic needs or optimization of parameters is an important factor in achieving maximum output. Therefore, in order to determine the best medium for production of increased fatty acid quantities, initial biomass optimization experiments were undertaken. Specifically, the recently developed Taguchi method (Joseph J and Piganatiells JR, *IIE Trans* 20:247-254, 1998), based on orthogonal arrays, was used in order to determine the optimum medium configuration for increased optical density (directly related to biomass production). In this instance, the Taguchi method was used to gain an understanding of the cumulative effects of the variables that pose an impact on biomass production. The effects of variations in nitrogen (yeast extract, peptone, L-glutamate), carbon (glucose) and salt concentration (artificial sea salt) had on biomass production. Therefore, a variety of liquid media were prepared with varying amounts of yeast extract, peptone and L-glutamate (0, 4, 10, 20, 50 g L^{-1}) as relates to varying amounts of glucose and sea salt solution (5, 40, 100, 160, 200 g L^{-1} and 0, 6, 20, 30, 40 g L^{-1}, respectively). Concentrations were calculated according to a L^{25} orthogonal array in such a way that nitrogen medium of choice was discerned through the use of signal to noise ratio (SNL) analysis at 48 and 120 hrs, using the following formula:

$$SNL = -10\log\left[\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}\right]$$

where, n = number of levels and y = yield (average OD_{600} from triplicate experiments).

339. Results from these experiments (which specifically target biomass considerations), as shown within figure 2 below, demonstrated that the rate of nitrogen utilization by ONC-T18 as relates to optical density (OD_{600}) was peptone, then yeast extract followed by L-glutamate. However, based on growth maxima the best nitrogen

sources for increase biomass production was yeast extract, then peptone, followed by L-glutamate. Furthermore, through the use of similar experiments with variations in glucose and salinity (sea salt concentration), the optimal and cheapest medium composition for the production of ONC-T18 biomass was within a medium comprising 2 g L^{-1} yeast extract, 8 g L-1 MSG, 60 g L^{-1} glucose and 6 g L^{-1} sea salt.

4. Example 4: Optimized production of docosahexaenoic acid (DHA) by strain ONC-T18

340. Media consisting of a nitrogen source (either peptone, yeast extract, L-glutamate (MSG) or combinations of these) and a carbon source (glucose), in a saline (artificial sea water) solution, were prepared in order to determine the best media composition for optimal biomass and DHA production in a similar manner to that described in Example 3 (shown in Table 4). After cultivation at 25°C and 130 rpms over 3 days, biomass, total fatty acid per liter of medium, percent content of fatty acids by weight, percent content of DHA in total fatty acids and the amount of DHA per liter of medium were determined via gas chromatography as per method described in example 1, and herein, and shown in Table 4 below.

341. In this case, DHA was confirmed by comparison to known standards of DHA using gas chromatography mass spectrometry and peak locking methods. Findings from experimental package ❶, where variations in both natural and organic forms of nitrogen were investigated, showed that the optimal media composition should contain between 4.0 and 6.0 g L^{-1} of both yeast extract and L-glutamate for optimal biomass and DHA production. Experimental package ❷, on the other hand, which investigated changes in the composition of sodium added to the medium showed optimal DHA production and biomass production when artificial sea salt is used. Moreover, experimental package ❸, in which the concentration of sodium within the medium was varied, depicted maxima for DHA and biomass production between 5 and 15% artificial seawater L^{-1} dH_2O. Results from experimental package ❹, where variations in glucose levels were evaluated, demonstrated that the range of 40 to less than160 g L^{-1} glucose translated into optimal biomass and DHA production. Finally, results of experimental package ❺ indicate that ONC-T18 produced equivalent values for both cellular biomass and DHA concentration, when glucose or glycerol were used as carbon sources.

Table 4: Results of DHA production optimization experiments with respect to variations in medium compositions.

	Exp No.	Carbon Sources		Salt types		Nitrogen Sources		Total fatty acids ($g\ L^{-1}$)	% fatty acids (wt %)	% content DHA (wt %)	Amount of DHA ($g\ L^{-1}$)
		Carbon source used	Amount added ($g\ L^{-1}$)	Salt mix used	Amount added (% salinity)	Yeast extract ($g\ L^{-1}$)	MSG ($g\ L^{-1}$)				
❶	401	Glucose	60.0	Sea salt	15.0	10.0	0.0	7.7	34.53	20.20	1.5579
	402	Glucose	60.0	Sea salt	15.0	8.0	2.0	10.0	44.01	17.09	1.7156
	403	Glucose	60.0	Sea salt	15.0	6.0	4.0	11.5	50.69	16.23	1.8624
	404	Glucose	60.0	Sea salt	15.0	4.0	6.0	16.9	69.07	24.19	4.0877
	405	Glucose	60.0	Sea salt	15.0	2.0	8.0	21.3	81.73	20.99	4.4752
	406	Glucose	60.0	Sea salt	15.0	0.0	10.0	0.15	1.97	28.81	0.0426
❷	407	Glucose	60.0	Sea salt	15.0	10.0	0.0	13.2	58.92	31.44	4.1362
	408	Glucose	60.0	NaCl	15.0	10.0	0.0	9.6	63.75	38.45	3.7009
	409	Glucose	60.0	$NaSO_4$	15.0	10.0	0.0	0.05	1.41	20.14	0.0109
❸	410	Glucose	60.0	Sea salt	5.0	10.0	0.0	14.6	59.23	31.44	4.6004
	411	Glucose	60.0	Sea salt	15.0	10.0	0.0	10.8	51.01	26.17	2.8144
	412	Glucose	60.0	Sea salt	37.5	10.0	0.0	15.9	69.32	25.32	4.0194
	413	Glucose	60.0	Sea salt	75.0	10.0	0.0	10.8	61.02	25.25	2.7356
	414	Glucose	60.0	Sea salt	100.0	10.0	0.0	11.8	68.21	24.02	2.8290
	415	Glucose	60.0	Sea salt	125.0	10.0	0.0	11.2	59.63	22.56	2.5256
❹	416	Glucose	5.0	Sea salt	15.0	10.0	0.0	0.63	5.21	29.18	0.1844
	417	Glucose	20.0	Sea salt	15.0	10.0	0.0	4.06	29.59	24.01	0.9752
	418	Glucose	40.0	Sea salt	15.0	10.0	0.0	9.91	59.39	23.88	2.3665
	419	Glucose	60.0	Sea salt	15.0	10.0	0.0	10.76	51.01	26.17	2.8144
	420	Glucose	100.0	Sea salt	15.0	10.0	0.0	12.79	69.50	31.55	4.0344
	421	Glucose	160.0	Sea salt	15.0	10.0	0.0	1.00	9.40	30.01	0.3013
❺	422	Glucose	5.0	Sea salt	15.0	10.0	0.0	0.62	12.74	29.86	0.1866
	423	Glycerol	5.0	Sea salt	15.0	10.0	0.0	0.52	18.84	35.07	0.1836

5. Example 5: Optimal time for harvesting of ONC-T18 for maximal DHA production

342. ONC-T18 was cultured under the same media composition and conditions as those shown within example 1. Of interest in this particular instance is the time at which ONC-T18 should be harvested in order to gain the maximal amounts of DHA, DPA and EPA as well as taking into account the time necessary to gain said amounts (see figure 3).

343. Time course experimental results showed that the optimal time for harvesting ONC-T18 for optimal DHA production within flask and bioreactor varied between 3 and 5 days, respectively.

6. Example 6: Analysis of Lipids Derived from ONC-T18

344. The total lipid fraction of ONC-T18 was extracted using a modified Bligh & Dyer method. Specifically, 2.0 g of dried cell biomass was rehydrated overnight at 4°C in 8 ml of distilled H_2O. 30 ml of methanol:chloroform (2:1 vol/vol) was added to mixture and gently shaken at 120 rpm for 20 min, with resultant supernatant decanted. Pellet was then resuspended in methanol:chloroform:H_2O (2:1:0.8 vol/vol/vol) and the process repeated with supernatants being pooled and moved to a separation funnel. 5 ml of chloroform and 5 ml of H_2O were then added to funnel resulting in the formation of a two-phase liquid system. After vigorous mixing within separation funnel, the chloroform layer was removed, concentrated under N_2 gas, resuspended in chloroform and stored at –20°C under analyzed. Approximately 1 µl of the total lipid fraction was spotted on multiple chromarods, separated and analyzed using an Iatroscan MK6 TLC/FID instrument.

345. Analysis of results shows that the fatty acid component which ONC-T18 produces under heterotrophic fermentation is almost entirely triglyceride (at least 95%) in nature. In addition to the neutral fatty acid fraction mentioned above. ONC-T18 also produces a discernable carotenoid and phospholipid fraction. On subsequent isolation of the phospholipid fraction, first via a 50% and then a 75% burn followed by solvent-based separation, it was determined that a large and complex phospholipid fraction was present. Results showed the present of phosphotidylcholine, phosphotidylserine and phosphotidic acid components within the sample.

7. Example 7: Production of antioxidants using the strain, ONC-T18

346. The eukaryote, ONC-T18 was cultured using conditions and medium as mentioned previously. The resultant cellular biomass after heterotrophic fermentation

is collected via centrifugation, filtration or settling. Cells were harvested by centrifugation at 3800 x g and washed with phosphate buffered saline. The cellular biomass (fresh or freeze-dried) was suspended in 10 x volume of acetone, agitated for 5 minutes at 200 rpm, centrifuged at 3800 x g for 5 minutes and concentrated to dryness by N_2 evaporation. The pigments were then immediately resuspended in a minimal amount of 10% acetone in hexane and stored at –20°C until HPLC analysis. Identification of carotenoid extracts was then carried out on an Agilent 1100 HPLC (Agilent, Palo Alto, CA, USA) equipped with a variable wavelength detector set at 470nm. Samples were injected through a Symmetry C18 guard column (Waters, Milford, MA, USA) to a Bondclone C18 reverse-phase column (Phenomenex, Torrance, CA, USA; 10 µm particles; 3.9 x 300 mm i.d.). The injection volume was 10 µl and a flow of 1.00 ml/min 10% acetone in hexane over a 25 minute period was used. Quantitative data of carotenoids was based on comparison of the peak area with known standards (in this case astaxanthin, canthaxanthin, β-cryptoxanthin, zeaxanthin, echinenone and β-carotene; ChromaDex, Santa Ana, CA, USA). In the absence of a known standard such as in the case of the carotenoid, phoenicoxanthin, the astaxanthin peak area was used to calculate its concentrations. Carotenoid identity was further confirmed via HPLC-MS using a Waters HPLC equipped with a photo-diode array (Waters model 996) leading into a Micromass ESI-Q-Tof Mass spectrometer (Waters, Milford, MA, USA). HPLC analysis of ONC-T18 subsequently revealed the presence of several antioxidant compounds (between 50 to 1250 mg kg^{-1}) within the cellular biomass. These compounds included the antioxidant carotenoids astaxanthin, zeaxanthin, canthaxanthin, echineone and beta-carotene in the range of 1 to 20 mg kg^{-1}, 0.25 to 10 mg kg^{-1}, 1 to 20 mg kg^{-1} 1 to 20 mg kg^{-1} and 1 to 200 mg kg^{-1}, respectively, as well as several unidentified flavenoid polyphenolic compounds.

8. Example 8: Comparison with Known Microorganisms

347. The ability of ONC-T18 to produce DHA, EPA and DPA was compared with that of known microorganisms. The amount of cellular biomass per liter of medium, the percent content of fats or fatty acids per dried cell biomass, the percent content of DHA, EPA and DPA in total fatty acids, and the amount of DHA, EPA and DPA obtained when DHA, EPA and DPA are produced by cultivation of *Thraustochytrium aureum* ATCC 34304, *Thraustochytrium* sp. ATCC 20891, *Thraustochytrium* sp. ATCC 20892, *Thraustochytrium roseum* ATCC 28210, *Thraustochytrium* sp. ATCC 26185, *Schizochytrium* sp. ATCC 20888, *Schizochytrium*

aggregatum ATCC 28209 and *Schizochytrium limacinum* MYA-1381, as well as when DHA, EPA and DPA are produced by cultivation of ONC-T18 according to the present invention.

Table 5: Comparison of the lipid production and biomass characteristics of several representative Thraustochytrid strains.

Microorganism	Cellular biomass amount ($g\ l^{-1}$)	Percent lipid content ($\%\ g^{-1}$)	Percent content of DHA ($\%\ g^{-1}$)	Percent content of EPA ($\%\ g^{-1}$)	Percent content of DPA ($\%\ g^{-1}$)	Total DHA ($g\ l^{-1}$)	Total EPA ($mg\ l^{-1}$)	Total DPA ($mg\ l^{-1}$)
Thraustochytrium sp ATCC 20891	1.8	no data	12	no data	no data	no data	no data	no data
Thraustochytrium sp ATCC 20892	3	7	35	no data	no data	0.07	no data	no data
Thraustochytrium sp ATCC 26185	2.3	no data	41.9	3.1	10	no data	no data	no data
T. aureum ATCC 34304	4-5	8-20	24-51	3.6-9.3	no data	0.1-0.5	0.0001	no data
T. roseum ATCC 28210	8-17	18-25	50	no data	no data	0.6-2.1	no data	no data
Schizochytrium sp. ATCC 20888	10.5	50	25-37	1.2	16.8	1.95	12.3	168.46
S. aggregatum ATCC 28209	1.4	1.7	6.0	6.1	no data	1	1	no data
S. limacinum SR21 MYA-1381	23-40	40-53.5	29.7-34	0.2-0.4	no data	3.0-7.2	0.08	no data
ONC-T18	25-55	45-80	24-34.2	0.1-2	6-10	4.6-13	0.2-0.8	0.9-3.8

348. As shown in Table 5, it is apparent that, when cultivation is carried out using ONC-T18 according to the present invention, the cellular biomass values per liter medium were extremely high as compared with the other strains tested. Moreover, according to the present invention, ONC-T18 has a very high percent content of lipids compared with the other strains mentioned above. Furthermore, according to the present invention, the percent content of DHA and DPA within ONC-T18 is extremely high, with EPA levels shown to be comparable to all strains

screened. Thus, it appears that ONC-T18 has the ability to produce large quantities of DHA, EPA and DPA under fermentation conditions as mentioned within example 1.

9. Example 9: Alternative Carbon Source information

349. ONC-T18 has been shown to grow preferentially on media where the main nitrogen sources are yeast extract, sodium glutamate and/or peptone and the main carbon source is D-glucose. As a result of detailed metabolic profiling of ONC-T18 it was noted that glycerol (carbon source) was also a viable alternative. Furthermore, fish oil processing waste streams containing glycerol were also tested for applicability as low-cost nutrient alternatives. Experiments using 200 ml media in 500 ml flasks, grown at 25°C for 3 days, 120 rpm in the case of the glycerol were undertaken. The glycerol content of two fish oil processing waste products, GWW (glycerol water wash) and GAW (glycerol acid wash), constituted 40% vol:vol of the 200 ml medium (adjusted to pH 6.5), while 6% glycerol was added to 200 ml medium (wt:vol) as control.

350. Analysis of these results has determined that the use of fish oil waste stream components, such as glycerol by-products, as carbon sources in large-scale fermentation of ONC-T18 while resulting in a reduced total fatty acid amount, represent a maintained DHA content within the microbial cells (figure 10).

Table 6: Fatty acid, biomass and glycerol content for alternative carbon source study.

	Percentage (%) fatty acid to total lipid content by weight					**TFA (mg g^{-1})**	**Glycerol (g L^{-1})**	**Biomass (g L^{-1})**
	AA	**EPA**	**DHA**	**DPA n-3**	**DPA n-6**			
6% Glycerol (wt:vol)	0.29	0.52	26.31	0.24	9.49	426.12	76.00	9.13
40% GAW (vol:vol)	0.37	1.32	19.69	0.42	6.36	294.55	68.59	5.94
40% GWW (vol:vol)	0.46	5.55	12.46	1.01	3.82	274.33	2.70	3.08

10. Example 10: Multiplier of dry cell weight

351. *Thraustochytrium* sp. ONC-T18 can be grown for the production of omega-3 oils in a variety of reactor configurations up to 100,000L. All fermentations begin with the preparation of a 10-20% final volume inoculum, which is used to establish the fermentation culture. Initial medium configurations comprise up to 6 g/L sea salt, 10 g/L nitrogen source and 60 g/L carbon source, with fed-batch addition of another 75 g/L carbon source after 24 to 36 hours of initial fermentation for an additional 72 to 96 hours and occurs within the temperature range 18-25°C. For example, using the medium 6 g/L sea salt, 2 g/L yeast extract, 8 g/L L-glutamate and 60 g/L D-glucose (with an addition 75 g/L added after 36 hours), grown at 25°C for 96 hours, ONC-T18 was able to produce 40 g/L dry cell weight (dcw), 80% (dcw) total fatty acid (TFA)/lipid fraction (between C14:0 and C24:0) and 30% (TFA) DHA. Similarly, it is possible to increase dry cell weight by multiplying both nitrogen and carbon media components to exact a similar multiplication effect on biomass without affecting either TFA or DHA contents. For example, using the medium 24 g/L sea salt, 8 g/L yeast extract, 32 g/L L-glutamate and 300 g/L D-glucose, grown at 25°C for 312 hours, ONC-T18 was able to produce 80 g/L dry cell weight (dcw), 60% (dcw) total fatty acid (TFA)/lipid fraction (between C14:0 and C24:0) and 38% (TFA) DHA.

11. Example 11: Growth of *Thraustochytrium* sp. ONC-T18 on various alternative, carbon (C) and nitrogen (N) sources and the effect on dry cell weight and lipids

352. Growth of *Thraustochytrium* sp. ONC-T18 on a variety of low-cost nitrogen and carbon sources was investigated. Specifically, 50 ml of ONC-T18 was cultured in 250 ml flasks containing 6 g/L artificial sea salts, for 72 hours at 25°C. Carbon and nitrogen source concentrations are shown below with 2 g/L of each nitrogen source listed, used in conjunction with 8 g/L of L-glutamate (with the exception of fish meal where 4 g was used). Carbon sources were switched as indicated. All experiments were performed in triplicate; all extractions for fatty acid methyl ester analysis were performed in triplicate along with triplicate GC injections.

353. Results indicate that *Thraustochytrium* sp. ONC-T18 produces optimal dry cell biomass (i.e. greater than the two control media) when grown on the nitrogen sources EMD yeast extract and fish meal. Conversely, lipid was found to be less than control, while DHA was optimal using corn steep liquor and EMD peptone. Finally,

the carbon source dextrose was found to increase lipid content, while fructose and dextrose producing high DHA content than the controls.

Table 7: Growth of *Thraustochytrium* sp. ONC-T18.

	Medium (salt 6 g/l, growth 72 hrs, 50 ml cultures)	C (g/l)	N (g/l)	dcw/l medium (g)	Lipid (mg/g)	Lipid (g/l)	DHA (mg/g)	DHA (g/l)	DHA (% lipid)
Nitrogen Sources	Corn Steep Liquor-MSG	60	10	11.36	371.97	4.33	111.64	1.244	29.72
	Cotton Seed-MSG	60	10	9.99	297.08	2.70	45.20	0.500	16.94
	EMD™ YE-MSG	60	10	15.49	343.90	5.02	70.68	0.786	20.70
	EMD™ YE	60	10	35.79	189.01	6.76	37.14	0.448	19.65
	EMD™ Peptone-MSG	60	10	11.70	379.48	4.19	83.50	0.926	23.33
	Sigma™ YE-MSG	60	10	9.77	257.86	3.28	54.60	0.618	19.69
	Sigma™ YE	60	10	10.39	341.98	3.53	58.30	0.629	17.01
	Fermtech™ YE-MSG	60	10	13.99	269.53	3.82	56.97	0.664	21.10
	Fermtech™ YE	60	10	17.07	243.23	4.15	48.01	0.530	19.74
	Fish meal (62% protein)	60	12	19.53	290.72	5.68	73.59	0.828	25.31
Carbon Sources	Fructose	60	10	14.57	498.54	8.09	96.97	1.070	21.55
	Dextrose	60	10	14.98	623.91	9.87	113.69	1.232	18.94
	Corn Dextrin	60	10	4.65	89.69	0.39	25.69	0.278	26.75
	Gelatin	60	10	7.09	31.87	0.13	11.86	0.127	27.70
	Starch (corn)	5	10	4.85	94.04	0.46	19.49	0.206	20.72
		30	10	3.13	90.07	0.28	23.78	0.256	26.40
	Starch (wheat)	5	10	8.03	86.96	0.47	17.62	0.185	17.76
		30	10	18.16	18.59	0.34	3.83	0.042	20.58
	Control medium (1)	60	10	16.92	487.59	8.25	70.87	0.768	13.25
	Control medium (2)	60	10	10.88	483.06	6.06	74.64	0.818	16.19

Abbreviations:

MSG = L-glutamate (sodium) YE = Yeast extract

12. Example 12: Extraction Techniques for isolation of total lipids and fractions

354. A variety of methods for the isolation of selected omega-3 oils were tested in order to determine optimal isolation efficiency. These methods included: the standard Bligh & Dyer method (Bligh & Dyer, *Can J. Biochem. Physiol.*, 37:912-917, 1959); the combined extraction and transesterification method used specifically with Thraustochytrid species allowing for processing of samples for rapid GC FAME analysis (Lewis et al., *J. Microbiol. Methods*, 43:107-116, 2000); extraction by simultaneous saponification (Cartens et al., *J. Am. Oil Chem. Soc.* 73:1025-1031, 1996); and solid phase extraction using silica gel columns which can selectively isolate triglycerides, diglycerides and monoglycerides (Pinkart et al., *J. Microbiol.*

Methods, 34:9-15, 1998; Bateman & Jenkins, *J. Agric. Food Chem.*, 45:132-135, 1997).

355. Specifically, 40 grams of dry cell weight *Thraustochytrium* sp. ONC-T18 biomass produced in a single fermentation run (see example 1) was divided into 0.44 g lots and used for each technique. All techniques were performed in triplicate with efficiencies analyzed using fatty acid methyl ester determination via FID-GC, again in triplicate with triplicate runs per sample. Results demonstrate that total fatty acid content might vary between individual methods with fluctuations most probably due to solvent:compound saturation, biomass disruption considerations and other physical condition considerations (e.g. temperature and time).

Table 8: Extraction Techniques for isolation of total lipids and fractions.

Bligh and Dyer

	DHA	EPA	C14:0	C14:1	C15:0	C16:0	C16:1	C18:1	C20:0	C20:4	C22:5	TFA
	mg omega-3 per gram of biomass (mg/g)											
1	104.39	4.25	36.28	5.82	113.94	77.27	4.92	44.01	1.13	1.67	28.86	430.99
2	136.75	5.45	46.51	7.40	142.96	98.38	6.07	56.49	1.40	2.19	37.92	552.98
3	134.59	4.78	42.51	6.91	128.54	87.01	5.20	51.10	1.30	2.10	35.98	532.91
Av.	**125.24**	**4.83**	**41.77**	**6.71**	**128.48**	**87.55**	**5.40**	**50.53**	**1.28**	**1.99**	**34.25**	**505.63**

Direct Transesterification

	DHA	EPA	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	C20:0	C20:4	C22:5	TFA
	mg omega-3 per gram of biomass (mg/g)											
1	104.39	4.24	36.39	5.42	112.94	75.27	5.42	44.01	1.13	1.67	28.86	420.99
2	89.83	4.54	34.81	5.60	103.04	73.43	5.56	42.85	0.98	1.87	25.35	392.88
3	101.64	4.25	37.16	5.98	106.94	75.98	5.35	43.95	1.11	1.78	26.46	410.65
Av	**98.65**	**4.34**	**36.12**	**5.67**	**107.64**	**74.89**	**5.44**	**43.60**	**1.07**	**1.77**	**26.89**	**408.17**

Simultaneous saponification

	DHA	EPA	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	C20:0	C20:4	C22:5	TFA
	mg omega-3 per gram of biomass (mg/g)											
1	204.85	6.75	46.55	8.56	182.26	134.81	8.73	105.04	2.16	3.20	66.68	785.25
2	188.51	6.17	47.64	9.32	208.29	121.25	10.35	95.80	2.53	2.89	61.41	770.14
3	198.25	6.12	47.21	9.65	207.71	136.51	9.58	98.50	2.41	3.10	63.58	782.54
Av	**197.20**	**6.35**	**47.13**	**9.18**	**199.42**	**130.86**	**9.55**	**99.78**	**2.37**	**3.06**	**63.89**	**779.31**

Solid phase extraction

	DHA	EPA	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	C20:0	C20:4	C22:5	TFA
	mg omega-3 per gram of biomass (mg/g)											
1	169.17	0.42	68.41	10.83	204.43	140.14	8.09	76.97	1.75	3.00	47.05	748.33
2	172.26	0.44	69.59	11.01	207.01	143.74	8.11	78.72	1.74	3.27	47.86	819.04
3	173.65	0.43	69.21	11.31	208.97	146.64	8.16	77.64	1.73	3.64	46.98	785.64
Av.	**171.69**	**0.43**	**69.07**	**11.05**	**206.80**	**143.51**	**8.12**	**77.78**	**1.74**	**3.30**	**47.30**	**784.34**

N.B: Each value listed above is the average of triplicate runs using FID-GC for FAME analysis

13. Example 13: Isolation and characterization of polyunsaturated fatty acid producting *Thraustochytrium* sp. ONC-T18: screening in order to identify strain and optimization of production therein.

a) Materials and Methods

(1) Isolation and Maintenance of Thraustochytrids

356. Seventy marine samples including: *Spartina alterniflora*, *Zostera marina* and sediment were collected in eastern Canadian coastal sites from Nova Scotia, Prince Edward Island, New Brunswick, Newfoundland and Labrador between July and August 2002. Samples were placed in 20 mL vials containing 10 mL of sterile 0.2 μm filtered natural seawater and 300 mg L^{-1} penicillin and 500 mg L^{-1} streptomycin. Suspensions were baited with sterile pollen (*Acer* sp.) and incubated for 48 hours at 18°C, according to (Bremer, *Marine Mycology - A Practical Approach,* Fungal Diversity Press, Hong Kong, pp 49-61 (2000)). Pollen grains were then transferred by loop and streaked onto B1 agar plates (1 g L^{-1} yeast extract, 1 g L^{-1} peptone, 10 g L^{-1} agar to 1 L natural seawater) containing antibiotics and incubated. Single, irregular, hyaline colonies made up of spherical or limaciform cells and atypical of either yeast or bacterial colonies were picked and sub-cultured at least three times on B1 plates for purity.

(2) Biomass production for fatty acid screening

357. To screen isolates for growth and fatty acid production, liquid medium was prepared using 0.2 μm filtered natural seawater containing 2 g L^{-1} peptone (BD, Franklin Lakes, NJ, USA) and 2 g L^{-1} yeast extract (BD, Franklin Lanes, NJ, USA), which was sterilized by autoclaving, followed by the addition of 5 g L^{-1}, 0.2 μm filter sterilized glucose (Sigma-Aldrich, St. Louis, MO, USA) (Bowles et al., *J Biotechnol* 70:193-202 (1999)). A 30 mL volume culture was inoculated by loop from an agar plate and grown for 4 days at 18°C on a shaker at 100 RPM. 5 mL of this culture was then used to inoculate a 95 ml culture incubated for a further 4 days (stationary phase). Cells were harvested by centrifugation at 4,500 RPM, rinsed with 5 mL of distilled water and re-centrifuged. The cell pellets were freeze dried, weighed and stored at -80°C prior to derivatisation for fatty acid analysis.

(3) Preparation of fatty acid methyl esters (FAME)

358. Fatty acid methyl ester (FAME) extraction was via the direct transesterification method, modified from Lewis et al. (*J Microbiol Meth.* 43:107-116

(2000)). Specifically, 20 mg of freeze dried material and 3 ml of transesterification reaction mix (methanol:hydrochloric acid:chloroform (10:1:1 vol/vol)) were added. Cells were vortexed for 10 seconds to ensure even dispersal of biomass and placed at 90°C for 120 minutes. Once transesterification was complete, samples were removed and allowed to cool to room temperature. Water (1 ml) was then added and vortexed for 10 seconds. FAMEs were then extracted via the addition of 3 x 2 ml aliquots of hexane:chloroform (4:1), vortexed for 10 seconds and allowed to sit until clear liquid separations were achieved.

(4) Gas chromatographic (GC) analysis of FAMEs

359. GC analysis of FAMES was carried out using two internal standards (200 µl each). One hexacosaenoic acid (C23:0) is added prior to transesterification and the other, nonadecaenoic acid (C19:0) added directly before analysis. Analyses was performed using an Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a 30 m x 0.32 m internal diameter (0.25 µm film thickness) OMEGAWAX 320 fused-silica capillary column (Sigma-Aldrich, St. Louis, MO, USA) and flame ionization detector (injection volume 1 µl, carrier gas H_2 with a constant flow of 5.0 ml min^{-1} and set at 250°C, split ratio 50:1 to FID detector at 275°C). Confirmation of FAME identity was performed using a Trace GC-DSQ mass spectrometer (Thermo Electron, Boston, MA, USA) and comparison of retention times for laboratory standards.

(5) Genetic identification

360. The genomic DNA was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer instructions. The oligonucleotide primers used in amplifying the 18S rRNA gene, were modified from Honda et al. (*J Eukaryot Microbiol.* 46:637-647 (1999)) namely T18S1F 5'-CAACCTGGTTGATCCTGCCAGTA-3' and T18S5R 5'-TCACTACGGAAACCTTGTTACGAC-3'. A 20-µl PCR reaction mixture contained 2U Biolase™ DNA polymerase (Bioline, Boston, MA, USA), 1 x NH_4 reaction buffer, 3 mM $MgCl_2$, 1M Betaine (Sigma-Aldrich, St Louis, MO, USA), 200 µM of mix PCR nucleotides (Promega, Madison, WI, USA), 1 µM of each forward and reverse primer (MWG Biotech., High Point, NC, USA) and 100 ng of genomic DNA template. After an initial denaturation step for 3 minutes at 94°C, PCR amplification was performed using a Eppendorf Master Cycle Gradient thermal cycler (Eppendorf, Westbury, NY, USA), using a program of 45 seconds at 94°C, 30 seconds at 64°C and 2 minutes at

72°C for 30 cycles, followed by a 10 minute final extension at 72°C. The PCR product was purified using MoBio UltraClean PCR Clean-up Kit (MoBio Laboratories Inc, Carlsbad, CA, USA) for direct sequencing (MWG Biotech., High Point, NC, USA) using primers FA2, FA3, RA1, R (Mo et al., *Mar Biol* 140:883-889 2002), T18S1F and T18S5R. The resulting sequences were aligned and compared to nucleotide sequences of similar microorganisms stored in GenBank (Benson et al., *Nucleic Acids Res* 33:D34-38 (2005)) using DS Gene (Accelrys, San Diego, CA, USA). A phylogenetic tree was subsequently generated using the Neighbor-Joining method (Saito and Nei, *Mol Biol Evol* 4:406-425 (1987)), with the statistical significance assessed using 1,000 bootstrap resamplings (Felsenstein, *Evolution* 39:783-791 (1985)).

(6) Identification of carotenoids

361. Cells were harvested by centrifugation at 3800 x g and washed with phosphate buffered saline. Then resuspended in 10 x volume of acetone (Sigma-Aldrich, St Louis, MO, USA), agitated for 5 minutes at 200 RPM, centrifuged at 3,800 x g for 5 mins and concentrated to dryness by N_2 evaporation. Followed by resuspension in a minimal amount of 10% acetone in hexane prior to HPLC analysis. Identifications were carried out on an Agilent 1100 HPLC (Agilent, Palo Alto, CA, USA) equipped with a variable wavelength detector set at 470nm. Samples were injected through a Symmetry C_{18} guard column (Waters, Milford, MA, USA) to a Bondclone C_{18} reverse-phase column (Phenomenex, Torrance, CA, USA; 10 μm particles; 3.9 x 300 mm i.d.). The injection volume was 10 μl and a flow of 1 ml min^{-1} 10% acetone in hexane over a 25 minute period was used. Carotenoid identity was further confirmed with mass spectrometry analysis (Micromass ESI-QTof MS, Waters, Milford, MA, USA). Quantitative data for each carotenoid was based on the development of a calibration curve using standards (astaxanthin, zeaxanthin, canthaxanthin, echinenone and β-carotene) and comparing peak area with defined concentrations.

(7) Fermentation optimization

362. The effect of carbon, nitrogen and sea salt on fatty acid and DHA production were examined using batch cultures in 250 ml Erlenmeyer flasks shaken at 130 RPM for 3 days at 25°C. Further cultivation studies were carried out using a Biostat® Bplus Twin 5L Bioreactor (Sartorius BBI Systems Inc., Betlehem, PA, USA). A 100 ml inoculum was used to inoculate 4.9L of medium in the bioreactor. Glucose

concentration was measured using the Glucose (HK) Assay Kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturers instructions. The media constituents and the conditions employed in the bioreactor are detailed with the relevant results.

b) Results

363. A collection and screening process was developed whereby members of the protist family Labyrinthulida, especially the genus, Schizochytrium and Thraustochytrium, were isolated using pollen-baiting and selective bacteriological media. This study, covering 20 unique collection sites dispersed throughout Atlantic Canada, produced 68 pure strains, identified microscopically. Selection of oleaginous strains, having more than 20% of their cell dry weight being fatty acids, was based upon results of GC PUFA profiling, biomass productivity, maximal TFA, DHA and to a lesser extent EPA concentrations (Fig. 11), according to the method of (Lewis et al., *J Microbiol Meth* 43:107-116 (2000)). Values for biomass, TFA and subsequent DHA and EPA productivities ranged from 100 to 2300 mg L^{-1}, 27.1 to 321.14, 5.18 to 83.63 and 2.97 to 21.25 mg g^{-1}, respectively (Fig. 11).

364. All isolates which grew in liquid medium (54 out of 68), produced major amounts of omega-3 polyunsaturated fatty acid, particularly DHA which comprised between 22 and 80% of the total C20 to C22 content of these cells (Fig. 11). This confirms previous findings, whereby thraustochytrids isolated from cold temperate environments have fatty acid profiles with DHA being up to 53% of the total fatty acid present (Bowles et al., *J Biotechnol* 70:193-202 (1999) and Huang et al., *Mar Biotechnol* 5:450-457 (2003)). Of particular interest is ONC-T18 which produces up to 90% of its C20 to C22 content as DHA which is approximately 35% of the total intracellular fatty acids. This DHA content was shown to be equivalent to those of several commercial production strains, such as Schizochytrium sp. ATCC 20888 (32%) and S. limacinum MYA-1381/SR21 (34%) (Barclay et al, *J Appl Phycol* 6:123-129 (1994) and Yokochi et al., *Appl Microbiol Biotechnol* 49:72-76, (2003)). Furthermore, all isolates synthesized eicosapentaenoic acid (EPA), varying between 2 and 20% w/w of total PUFAs identified (Fig. 11). In addition to the omega-3 oils produced, approximately 80% of all isolates synthesized the omega-6 PUFAs, arachidonic acid (AA) or docosapentaenoic acids (DPA), at concentrations varying between 1 and 18% and 3 and 7% w/w, respectively (Fig. 11).

365. Huang et al. (*Mar Biotechnol* 5:450-457 (2003)) suggested that for thraustochytrids isolated from the tropical coastal waters of Japan and Fiji, five

polyunsaturated fatty acid profiles could be described, namely DHA/DPA (n-6), DHA/DPA/EPA, DHA/EPA, DHA/DPA/EPA/AA and DHA/DPA/EPA/AA/docosatetraenoic acid (Huang et al., *Mar Biotechnol* 5:450-457 (2003)). In the case of this collection of thraustochytrids, isolated from the temperate waters of Atlantic Canada, four PUFA profiles could be determined, three of which are identical to those mentioned above, namely DHA/DPA/EPA at 7.4% of collection, DHA/EPA at 13% of collection and DHA/DPA/EPA/AA, 74%, with a forth comprising a mixture of DHA/EPA/AA at 5.6%.

366. Through direct sequencing of the 18S rDNA gene, ONC-T18 was positively identified as a member of the Thraustochytrid family (GenBank Accession Number: DQ374149). Phylogenetic analysis indicated that ONC-T18 formed a unique group (97.5% identity) with Thraustochytrium striatum T91-6 (Fig. 12) (Leander and Porter, Mycologia 93:459-464 (2001)). While Thraustochytriidae sp. MBIC 11093, N1-27 and Thraustochytrium sp. CHN-1, collected from the coastal tropical waters of Japan, and found to be significant producers of DHA (Carmona et al., *Biosci Biotechnol Biochem* 67:884-888 (2003) and Huang et al., *Mar Biotechnol* 5:450-457 (2003)), were shown to be 96, 95.5 and 94.5% similar, respectively. Genetic diversity is quite low between all members of the Thraustochytriidae shown in figure 12, ranging from 97.5–91.0% similarity throughout. Yet, these species are globally distributed, with two-thirds isolated from the tropical coastal waters of Japan, China and Israel and the remaining from temperate waters off America, Europe and Canada.

367. The fatty acid profile of ONC-T18 included high contents of C22 PUFA, very low levels of C18 and C20 FA, and the occurrence of odd-chain saturated fatty acids (15:0 and 17:0), similar to that of Schizochytrium sp. KH105 or S. limacinum SR21. Furthermore, analysis of carbon and nitrogen utilization profiles for strains ONC-T18, SR21 and KH105 showed a similar pattern of assimilation. The content of n-6 DPA in strain ONC-T18 ranged from 6-10%, which seems to be extremely high when considering the limited occurrence of n-6 DPA in the biosphere. Similar levels of n-6 DPA were reported however, by Nakahara et al. (*J Am Oil Chem Soc* 73:1421-1426 (1996)) in Schizochytrium sp. SR21 (6-10%) and Ellenbogen et al. (*Comp Biochem Physiol* 29:805-81 (1969)) in T. aureum (9.5%) and T. roseum (6.6%).

368. Analysis of the fatty acid profile of ONC-T18 under three different culture configurations: (1) agar plate; (2) conical flask and (3) bioreactor and grown on the same medium (Fig. 13), shows a decrease in the diversity of PUFAs present and an

overall increase in TFA from agar plate to bioreactor. Specifically, agar plates exhibited an array of PUFAs, while the flask and bioreactor grown cultures were dominated by one or two intermediates (Fig. 13). Compared to Thraustochytrium aureum, which grew better in flask culture than in a stirred tank fermenter (Ilda et al., *J Ferment Bioeng* 81:76-78 (1996)), ONC-T18 grew better in a bioreactor. This result is in agreement with that of (Nakahara et al., *J Am Oil Chem Soc* 73:1421-1426 (1996)), who found that Schizochytrium sp. SR21 showed high resistance to mechanical stirring, and therefore thrived under bioreactor conditions.

369. Furthermore, carotenoid pigments were found to be produced in plate, flask and bioreactor fermentations of Thraustochytrium sp. ONC-T18, resulting in a pale orange discoloration. Production of these antioxidants is maximal within bioreactor fermentations concurrently with fatty acid production. Moreover, through the use of HPLC mass spectrometry, it was determined that these antioxidant compounds were identified as astaxanthin, zeaxanthin, canthaxanthin, echineone and β-carotene (Fig. 14), being conjugated to various PUFAs. Similar results were reported amongst members of the thraustochytid group of protists. Specifically, Schizochytrium aggregatum was shown to produce echinenone and canthaxanthin (Valadon, *Trans Br Mycol* Soc 67:1-15 (1976)), while Carmona et al. (*Biosci Biotechnol Biochem* 67:884-888 (2003) and Huang et al. (*Mar Biotechnol* 5:450-457 (2003)) demonstrated the production of astaxanthin, echinenone, canthaxanthin, phoenicoxanthin (not zeaxanthin as in ONC-T18) and β-carotene by Thraustochytrium sp. CHN-1, a close relative of ONC-T18 (Fig. 12). In this study, concentrations of these carotenoids were found to be an order of magnitude less than those of CHN-1 with the major compound being β-carotene, rather than astaxanthin. Thus, within Thraustochytrium spp., PUFA and carotenoid production can be linked so that the storage fats being produced may be protected from oxidation.

370. Previously, it has been determined that the relative amounts of the principal fatty acid components (myristic, palmitic and oleic acids) may be altered somewhat by changing the growth conditions of the culture (Ilda et al., *J Ferment Bioeng* 81:76-78 (1996)). In this way, one can manipulate the final fatty acid composition and hence, physical properties of the desired PUFA in a controlled fashion during fermentation (Sijtsma et al., *Recent Res Devel Microbiol* 2:219-232 (1998)). In an attempt to limit the factors inhibiting both biomass and omega-3 PUFA

production in ONC-T18, carbon, nitrogen and sea salt components in nutrient media were manipulated (Table 9), along with duration of culture (Fig. 15) .

Table 9: Mean biomass production (SD ≤ 15%), total fatty acid (TFA) and DHA content of *Thraustochytrium* sp. ONC-T18.

Glucose ($g L^{-1}$)	Biomass ($g L^{-1}$)	TFA (% biomass)	DHA (% TFA)	DHA ($g L^{-1}$)
5	12.13	5.21	29.18	0.18
20	13.73	29.59	24.01	0.98
40	16.69	59.39	23.88	2.37
60	21.08	51.01	26.17	2.81
100	18.40	69.49	31.55	4.03
160	10.68	9.40	30.01	0.30

YE ($g L^{-1}$)	MSG ($g L^{-1}$)	Biomass ($g L^{-1}$)	TFA (% biomass)	DHA (% TFA)	DHA ($g L^{-1}$)
10	0	22.33	34.53	20.20	1.56
8	2	22.81	44.00	17.52	1.72
6	4	22.64	50.69	16.23	1.86
4	6	24.46	69.07	24.19	4.09
2	8	26.09	81.73	20.99	4.47
0	10	7.50	1.97	28.81	0.04

Sea salt ($g L^{-1}$)	Biomass ($g L^{-1}$)	TFA (% biomass)	DHA (% TFA)	DHA ($g L^{-1}$)
2	24.70	59.23	31.44	4.60
6	21.08	51.01	26.17	2.81
15	22.90	69.32	25.32	4.02
30	17.76	61.02	25.25	2.74
40	17.27	68.21	24.02	2.83
50	18.77	59.63	22.56	2.53

371. Within this study, as the concentration of nitrogen decreased, total fatty acid content increased, with the highest total fatty acid content (approximately 80%) obtained at 1% concentration of yeast extract and or monosodium glutamate (w/v). Cultures with a low nitrogen concentration, however, also limited cell growth and hence total fatty acid production. Optimal production in this experiment was obtained using 8 g L^{-1} monosodium glutamate and 2 g L^{-1} yeast extract, producing 26.1 g L^{-1} biomass and 4.5 g L^{-1} DHA (Table 9). Furthermore, increases in carbon up to 100 g L^{-1} effectively increased DHA yield, this is in agreement with results obtained for Schizochytrium sp. SR21 (Yokochi et al., *Appl Microbiol Biotechnol* 49:72-76, (2003)) and contrary to those shown in T. aureum where glucose concentrations above 10 g L^{-1} were inhibitory (Ilda et al., *J Ferment Bioeng* 81:76-78 (1996)). Maximum DHA yields of more than 4.0 g L^{-1} were obtained in glucose medium, with yields more than 5 times that of T. aureum (Bajpai et al., *J Am Oil Chem Soc* 68:509-

514 (1991)) and T. roseum (Li and Ward, *J Ind Microbiol* 13:238-241 (1994)) and comparable to that of Schizochytrium sp. SR21 and KH105 (Aki et al., *J Am Oil Chem Soc* 80:789-794 (2003)). Finally, ONC-T18 exhibited classical euryhaline abilities, being able to withstand salinities ranging from 2.0 to 50.0 g L^{-1}, resulting in biomass productivity of 25-30% variability (Table 9). In the same experiment DHA g L^{-1} values were found to vary up to 45% between optimal at 4.6 g L^{-1} and minimal at 2.5 g L^{-1} (Table 9).

372. The biomass, TFA and DHA produced by ONC-T18 over a 168 h period in a 5 L bioreactor are presented in Figure 15. The growth curve depicted is typical of several achieved under identical conditions. Maximum biomass production was reached after 120 h, close to the point of carbon source (i.e. glucose) depletion. This was also the point at which total fatty acid content of the biomass reached a maximum at around 70% biomass. Interestingly, after only 24 h of cultivation, DHA content spiked to 30% total fatty acid, thereafter remaining constant at 20-25%. These results are consistent with those of other fatty acid producing Thraustochytid strains, yet there is disparity with regards to the rate at which these reactions occur.

c) Discussion

373. Previously most studies of Labyrinturomycota identified strains which are unable to store total fatty acid in amounts greater than 20% of biomass. For example, prior to the isolation of Schizochytrium sp. SR 21 which is able to accumulate up to 50% of biomass as fat, T. aureum was the best accumulator at 20% (Bajpai et al., *J Am Oil Chem Soc* 68:509-514 (1991)). ONC-T18, on the other hand, is able to accumulate up to 80% of its biomass as lipid.

374. For oleaginous micro-organisms such as ONC-T18 to accumulate oil, it typically should be grown in a culture medium with a limited amount of nitrogen (usually exhausted after 24 to 36 h) and abundant amounts of a carbon source. Once the nitrogen is depleted, the oleaginous microbes continue to assimilate the carbon source but are no longer able to undergo cell division due to a lack of nitrogen (thus preventing protein and nucleic acid synthesis). The result being the conversion of these carbon sources (i.e. sugars such as glucose) into storage oils. In this regard, ONC-T18 is considered to grow more slowly than other Thraustochytrid strains, such as G13 (Bowles et al., *J Biotechnol* 70:193-202 (1999) and Huang et al., *Mar Biotechnol* 5:450-457 (2003), yet it produces DHA at faster rates and demonstates a unique ability to incorporate elevated amounts of total fatty acids. Finally, the ability

of ONC-T18 to grow at very low salt concentrations with both high biomass and total fatty acid productivity is remarkable. Lending itself well to scale up by negating the corrosive nature of salt water on industrial fermentation equipment.

14. Example 14: Compound (fatty acid antagonist) supplementations

a) Sethoxydim supplementation

375. Media containing 25 g/l sea salt and 1g/l yeast extract was autoclaved at 120℃ for 20 minutes. Sterilized glucose was added to the media at a concentration of 9 g/l. Sethoxydim (Supleco) was dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml was then added to the media at to represent a final concentration of 100 □M. 1 ml of a 24 hour pre-culture of ONC-T18 that had been cultured in the same media was added. Culturing was carried out in a shaker (130 rpm) for four days at room temperature. After the incubation period, cells were harvested by centrifugation at 4300 rpm for 10 minutes. The cell pellets were then freeze-dried, weighed and the oil was extracted from the cells.

376. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced plus the substrate added) and then multiplying by 100. The Results are shown in Figure 11. The results also showed that sethoxydim exposure resulted in an increase in the content and proportion of DHA accumulated in ONC-T18.

b) Example 2: Cerulenin supplementation

377. Media containing 6g/l sea salt, 2g/l yeast extract, and 8g/l L-glutamic acid monosodium salt hydrate was autoclaved at 120°C for 20 minutes. Sterilized D+-glucose was then added to this media at a concentration of 40g/l. Cerulenin dissolved in ethanol at a concentration of 5 mg/ml was added to 250 ml flask and the solvent was evaporated for 1 hour prior to adding the media to the flask. The amount of

cerulenin in the flask represented a final concentration of 20 mg/l. ONC-T18 was then added to the medium from a plate culture and culturing occurred in a shaker (130 rpm) at room temperature for four days. After the incubation period, cells were harvested by centrifugation at 4300 rpm for 10 minutes, washed once with distilled water, and then centrifuged again at 4300 rpm for 10 minutes. The cell pellets were then freeze-dried, weighed and the oil was extracted from the cells.

378. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then multiplying by 100. Results are shown in Figure 11. The results also show that cerulenin exposure resulted in an increase in the content and proportion of DHA accumulated in ONC-T18.

c) Example 3: Oleic acid supplementation

379. Media containing 6 g/l sea salt, 2g/l yeast extract, and 8g/l L-glutamic acid monosodium salt hydrate was autoclaved at 120℃ for 20 minutes. Sterilized D+-glucose wad then added at a concentration of 5g/l or was not added to the media . Oleic acid was added to the media to represent a final concentration of 2 ml/l. ONC-T18 was then added to the media from a one day old inoculum that had been cultured in Windust media (5 g/l yeast extract, 5 g/l peptone, 40 g/l D(+)-glucose, 1.25 ml/l Windust trace elements, 1.25 ml/l Windust vitamins , 40g/l sea salt; (Windust trace elements: 5 g/l $NaH_2PO_4.H_2O$, 3.15 g/l $FeCl_3.6H_2O$, 4.36 g/l $Na_2EDTA.2H_2O$, 0.6125 mg/l $CuSO_4.5H_20$, 0.0597g/l $Na_2MoO_4.2H_2O$, 0.022 g/l $ZnSO_4.7H_20$, 0.01g/l $CoCl_2.6H_2O$, 0.18 g/l $MnCl_2.4H_2O$, 13 µg/l H_2SeO_3, 2.7 mg/l $NiSO_4.6H_2O$, 1.84 mg/l Na_3VO_4, 1.94 mg/l K_2CrO_4), (Windust vitamins: 1 mg/l vitamin B12, 1 mg/l biotin, 0.20 g/l thiamine HCl)). Culturing occurred in a shaker (130 rpm) for 3 days at room temperature. After the incubation period, cells were harvested by centrifugation at

4300 rpm for 10 minutes, washed once with distilled water, and then centrifuged again at 4300 rpm for 10 minutes. The cell pellets were then freeze-dried, weighed and the oil was extracted from the cells.

380. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then multiplying by 100. Results are shown in Figure 11. The results also show that oleic acid supplementation resulted in an increase in the content and proportion of DHA accumulated in ONC-T18.

d) Example 4: Capsaicin supplementation

381. Media containing 6 g/l sea salt and 10g/l yeast extract was autoclaved at 120 ℃ for 20 minutes. Sterilized D(+)- glucose was then added to the media at a concentration of 40g/l. 50 ml of this media was then put into a 250 ml Erlenmeyer flask. Capsaicin (Sigma) was dissolved in ethanol at a concentration of 10 g/l and was added to the media to represent a final concentration of 0.1 g/l. ONC-T18 was added to the medium from a plate culture and cultured in a shaker (130 rpm) at room temperature for four days. After the incubation period, cells were harvested by centrifugation at 4300 rpm for 10 minutes, washed once with distilled water, and then centrifuged again at 4300 rpm for 10 minutes. The cell pellets were then freeze-dried, weighed and the oil was extracted from the cells.

382. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and

allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then multiplying by 100. The results are shown in Figure 11. The results also show that capsaicin exposure resulted in a decrease in the content of C16:0 and increase the content of C16:1 in ONC-T18.

e) Example 5: Toluic Acid addition

383. Media containing 20 g/l sea salt and 10g/l yeast extract was autoclaved at 120℃ for 20 minutes. Sterilized D(+)-glucose was then added to the media at a concentration of 20g/l. 50 ml of this media was then put into a 250 ml Erlenmeyer flask and toluic acid (Sigma) dissolved in ethanol at a concentration of 20g/l was added to the media to represent a final concentration of 0.2 g/l. ONC-T18 was added into the medium from a plate culture and cultured in a shaker (130 rpm) at room temperature for four days. After the incubation period, cultured cells were recovered by centrifugation at 4300 rpm for 10 minutes, washed once with distilled water, and re-centrifuged. The cell pellets were freeze-dried, weighed and the oil was extracted from the cells. (Example 19)

384. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then multiplying by 100. The Results are shown in Figure 11. The results also show that toluic acid resulted in an increase the proportion of EPA and DPA n-3 and a decrease in the proportion of DPA (n-6) and DHA- possibly a Δ4-desaturase inhibitor in ONC-T18.

f) Example 6: Addition of Norflurazon

385. 50 ml of media containing 25 g/l sea salt and 1g/l yeast extract was autoclaved at 120 ℃ for 20 minutes. Sterilized D(+)- glucose was then added to the media at a concentration of 9g/l. 50 ml of this media was then put into a 250 ml Erlenmeyer flask. Norflurazon (Supleco) dissolved in dimethyl sulfoxide at a concentration of 20 g/l was then added to the media at to represent a final concentration of 0.2 g/l. 1 ml of a 24 hour pre-culture (inoculum) of ONC-T18 that had been cultured in the same media (25 g/l sea salt, 1 g/l yeast extract, 9g/l glucose) was added. Culturing was carried out in a shaker (130 rpm) for four days at room temperature. After the incubation period, cells were harvested by centrifugation at 4300 rpm for 10 minutes, washed once with distilled water, and then centrifuged again at 4300 rpm for 10 minutes. The cell pellets were then freeze-dried, weighed and the oil was extracted from the cells.

386. The lipids were extracted and derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC). Transesterification and extraction was done using 200 mg freeze dried cells, with C19:0 as internal standard, added transesterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) mixed and heated at 90°C for 2 hours, then allowed to cool at room temperature. FAMEs were extracted by adding 1 ml water, and 2 ml hexane: chloroform (4:1), and allow organic and aqueous phases to separate. The organic layer was extracted and treated with 0.5 g of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated under a stream of argon. The FAMEs were re-suspended in 5 ml of iso-octane and analyzed by GC-FID. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then multiplying by 100. The Results are shown in Figure 11. The results also show that norflurazon exposure results in an increase in the content and proportion of DHA accumulated in ONC-T18.

What is claimed is:

1. A method of preparing a lipid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.
2. The method of claim 1, further comprising isolating the lipid composition.
3. The method of claim 1, wherein the ratio of omega-3 and omega-6 fatty acids to omega-9 fatty acids is increased.
4. The method of claim 3, wherein the overall fatty acid content is unaltered.
5. The method of claim 1, wherein the oil producing microbe is a diatom, a microalgae, a fungi, a bacterium, a protist.
6. The method of claim 1, wherein the oil producing microbe has an 18S sequence, wherein the 18S sequence has at least 94% identity to the sequence set forth in SEQ ID NO:1
7. The method of claim 1, wherein the oil producing microbe has an 18S sequence, wherein the 18S sequence has at least 80% identity to the sequence set forth in SEQ ID NO:1
8. The method of claim 1, wherein the oil producing microbe is from the phylum Labyrinthulomycota.
9. The method of claim 1, wherein the oil producing microbe is from the class Labyrinthulomycetes.
10. The method of claim 1, wherein the oil producing microbe is from the subclass Thraustochytridae.
11. The method of claim 1, wherein the oil producing microbe is from the order Thraustochytriales.
12. The method of claim 1, wherein the oil producing microbe is from the family Thraustochytriaceae.
13. The method of claim 1, wherein the oil producing microbe is from the genus *Thraustochytrium*.
14. The method of claim 1, wherein the oil producing microbe is *Thraustochytrium* sp.

15. The method of claim 1, wherein the oil producing microbe is *Thraustochytrium aureum*.

16. The method of claim 1, wherein the oil producing microbe is *Thraustochytrium roseum*.

17. The method of claim 1, wherein the oil producing microbe is *Thraustochytrium striatum*.

18. The method of claim 1, wherein the oil producing microbe is from the genus *Schizochytrium*.

19. The method of claim 1, wherein the oil producing microbe is *Schizochytrium* sp.

20. The method of claim 1, wherein the oil producing microbe is from the family Thraustochytriaceae and has ATCC accession number PTA-6245, 20888, 20889, 20890, 20891, or 20892.

21. The method of claim 1, wherein the oil producing microbe is ONC-T18.

22. The method of claim 1, wherein the fatty acid production antagonist is selected from the group consisting of oleic acid, cerulenin, toluic acid, curcumin, cyclopropene, alkyl gallate, propyl gallate, sesame oil, peanut oil, canola oil, pyridazinone, norflurazon, diphenylamine, sethoxydim, and capsaicin.

23. A delivery device comprising the lipid composition of claim 1.

24. The delivery device of claim 23, wherein the delivery device comprises a microcapsule, a microsphere, a nanosphere or nanoparticle, a liposome, a noisome, a nanoerythrosome, a solid-liquid nanoparticle, a leuprolide, a gel, a gel capsule, a tablet, a lotion, a cream, a spray, an emulsion, or a powder.

25. A microcapsule, comprising an agglomeration of primary microcapsules and a loading substance, each individual primary microcapsule having a primary shell, wherein the loading substance comprises the composition of claim 1, and is encapsulated by the primary shell, and wherein the agglomeration is encapsulated by an outer shell.

26. The microcapsule of claim 25, wherein the primary shell and/or outer shell comprises a surfactant, gelatin, polyphosphate, polysaccharide, or a mixture thereof.

27. The microcapsule of claim 25, wherein the loading substance comprises oil from Thraustochytrium, Schizochytrium, or a mixture thereof.

28. The microcapsule of claim 25, wherein the outer shell has an average diameter of from about 1 μm to about 2,000 μm.

29. The microcapsule of claim 25, wherein the outer shell has an average diameter of from about 20 μm to about 1,000 μm.

30. The microcapsule of claim 25, wherein the outer shell has an average diameter of from about 30 μm to about 80 μm.

31. The microcapsule of claim 25, wherein the primary shell has an average diameter of from about 40 nm to about 10 μm.

32. The microcapsule of claim 25, wherein the primary shell has an average diameter of from about 0.1 μm to about 5 μm.

33. The microcapsule of claim 25, wherein the loading substance is from about 20% to about 90% by weight of the microcapsule.

34. The microcapsule of claim 25, wherein the loading substance is from about 50% to about 70% by weight of the microcapsule.

35. A nutritional supplement comprising the composition of claim 1, the delivery device of any of claims 23-24, or the microcapsule of any of claims 25-34.

36. The nutritional supplement of claim 35, wherein the supplement is in the form of a tablet, gel-cap, capsule, liquid, or syrup.

37. A foodstuff comprising the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, or the nutritional supplement of any of claims 35-36.

38. The foodstuff of claim 37, wherein the foodstuff is a baked good, a pasta, a meat product, a frozen dairy product, a milk product, a cheese product, an egg product, a condiment, a soup mix, a snack food, a nut product, a plant protein product, a hard candy, a soft candy, a poultry product, a processed fruit juice, a granulated sugar, a sauce, a gravy, a syrup, a nutritional bar, a beverage, a dry beverage powder, a jam or jelly, an infant formula, or a baby food.

39. The foodstuff of claim 37, wherein the foodstuff is a fish product, a companion pet food, a livestock or an aquaculture feed.

40. The foodstuff of claim 37, wherein the foodstuff is bread, tortillas, cereal, sausage, chicken, ice cream, yogurt, milk, salad dressing, rice bran, fruit juice, a dry beverage

powder, rolls, cookies, crackers, fruit pies, or cakes.

41. A method of delivering a composition to a subject, comprising administering to the subject the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

42. The method of claim 41, wherein the subject is a mammal.

43. The method of claim 41, wherein the subject is a human.

44. A use of a microcapsule of any of claims 25-34 and to prepare a medicament for delivering a loading substance to a subject.

45. A method of lowering cholesterol levels, triglyceride levels, or a combination thereof in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

46. A method of supplementing essential trace elements in a subject, the method comprising the step of administering to the subject an effective amount of a the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40, wherein the composition, delivery device, microcapsule, supplement, and foodstuff comprises an essential trace element.

47. A method improving insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

48. A method of reducing hyperglycemia in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

49. A method of reducing hypercholesterolemia in a subject, comprising the step of

administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

50. A method of reducing body fat in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

51. A method of promoting weight loss in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

52. A method of treating or preventing diabetes in a subject, comprising the step of administering to the subject an effective amount of the composition of claim 1, the delivery device of any of claims 23-24, the microcapsule of any of claims 25-34, the nutritional supplement of any of claims 35-36, or the foodstuff of any of claims 37-40.

53. A pharmaceutical formulation comprising a the composition of claim 1, the delivery device of any of claims 23-24, or the microcapsule of any of claims 25-34, and a pharmaceutical carrier.

54. A pharmaceutical formulation comprising the composition of claim 1.

55. A method of preparing a carotenoid composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

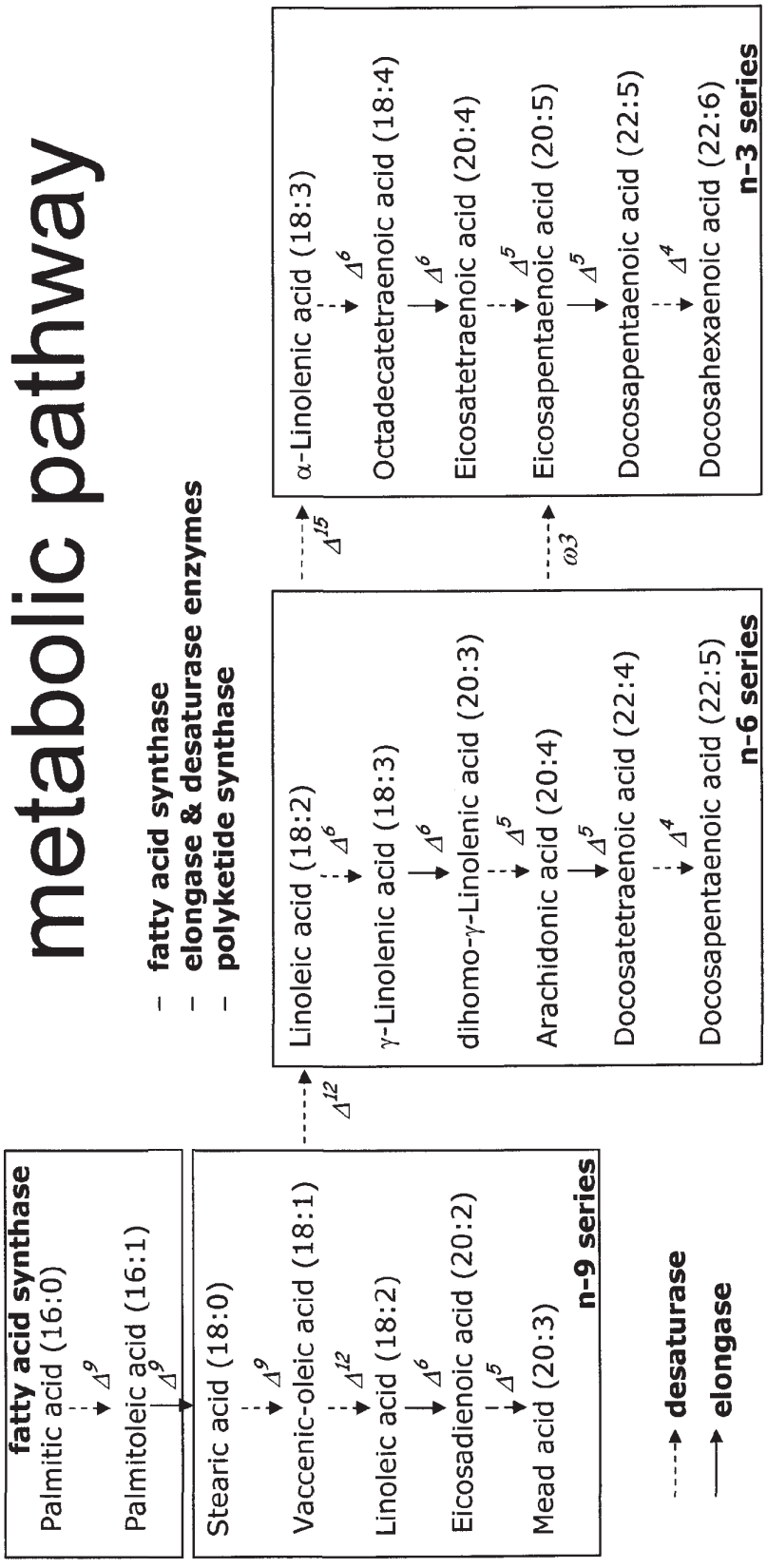
56. The method of claim 55, further comprising isolating the carotenoid composition.

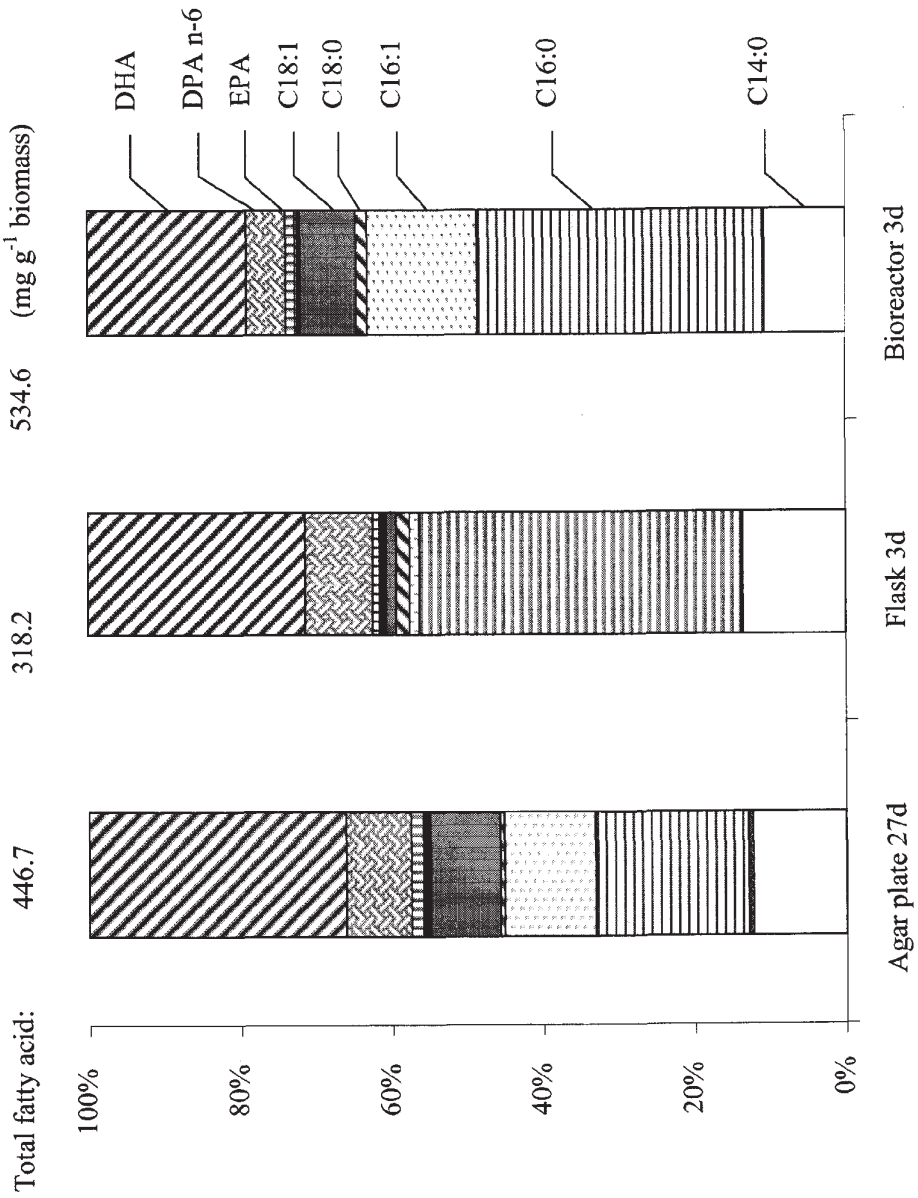
57. A pharmaceutical formulation comprising the composition of claim 55.

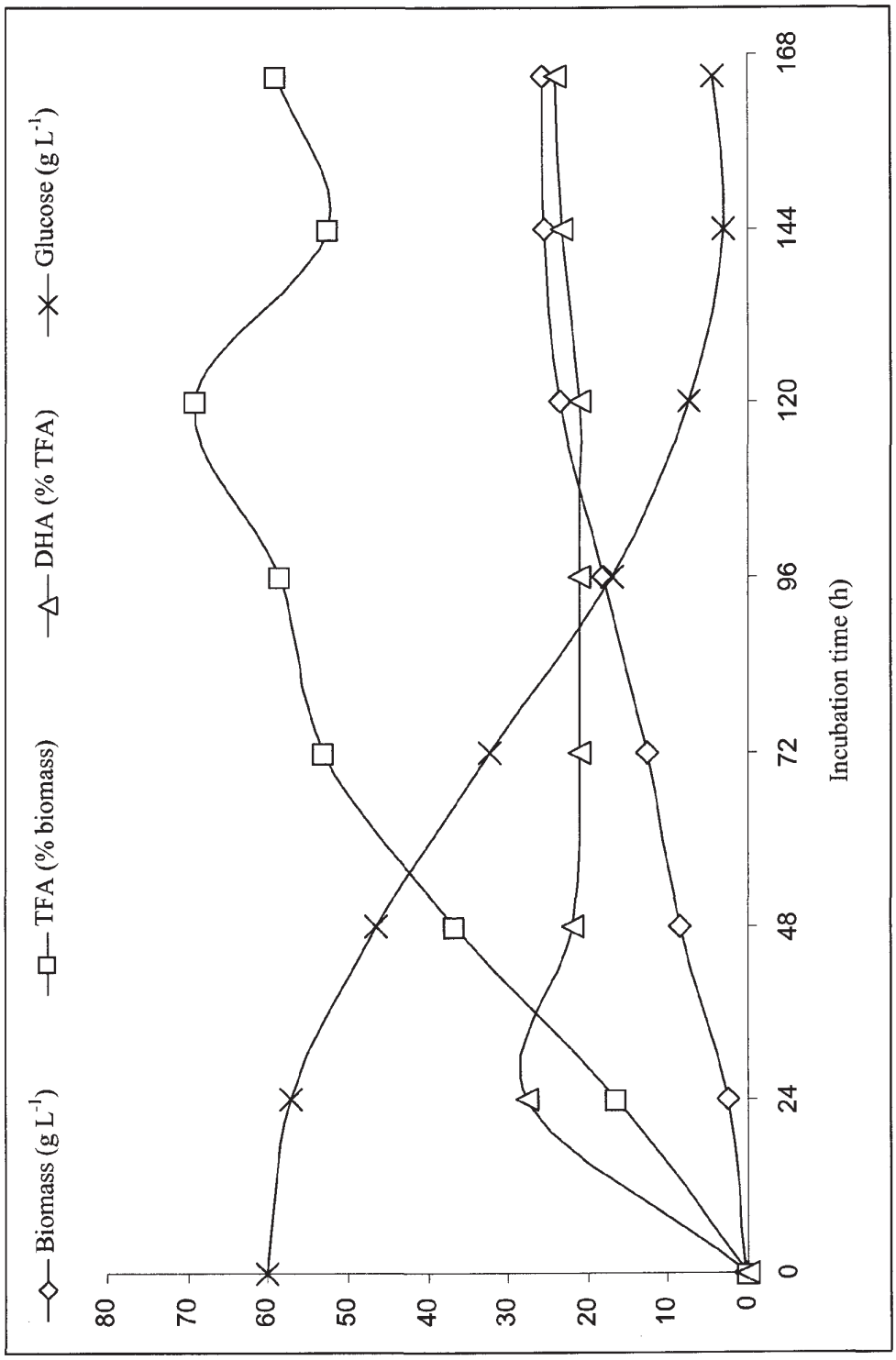
58. A method of preparing an antioxidant composition, the method comprising: culturing an oil producing microbe in a heterotrophic medium comprising a fatty acid production antagonist.

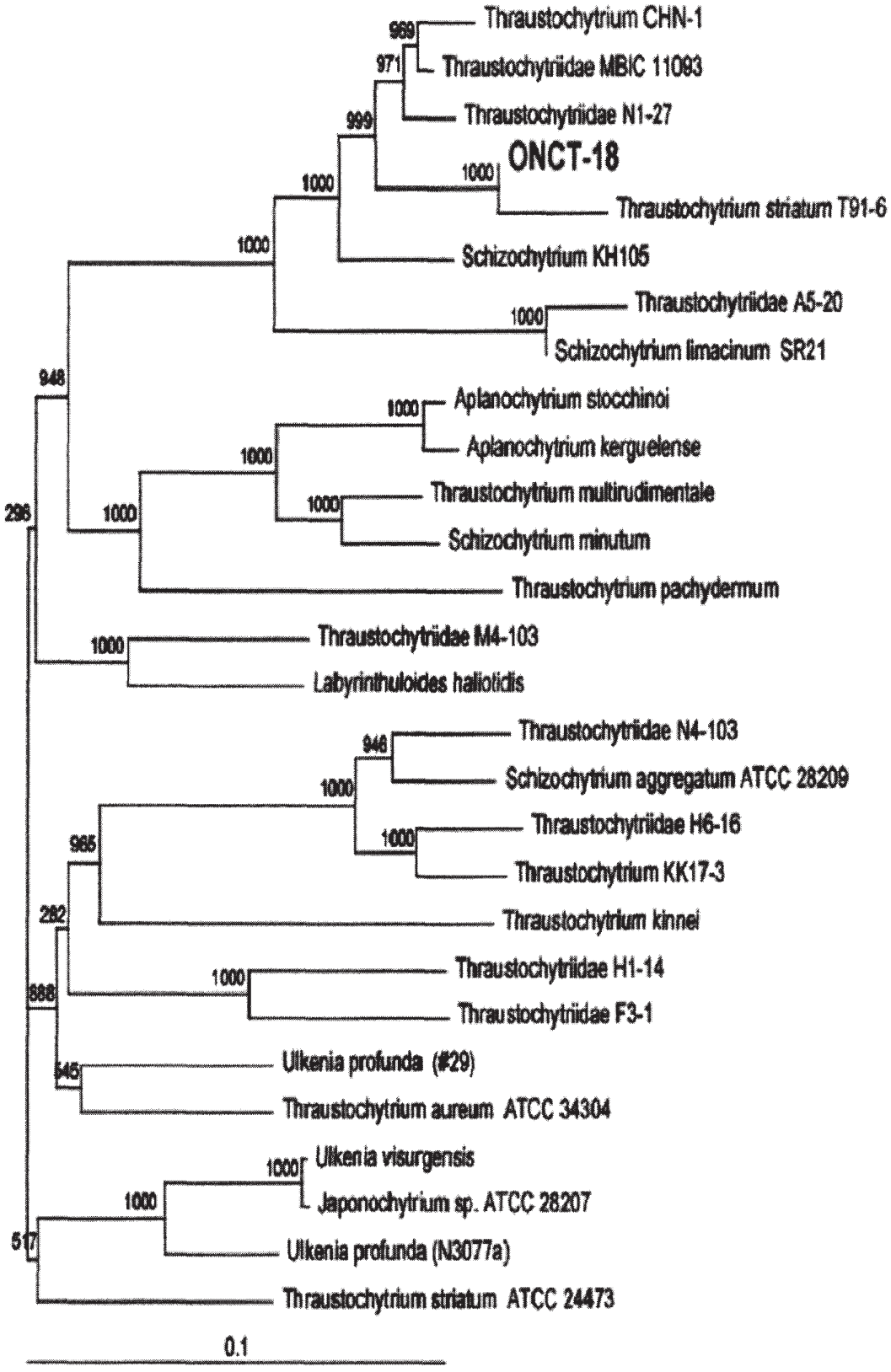
59. The method of claim 58, further comprising isolating the antioxidant composition.

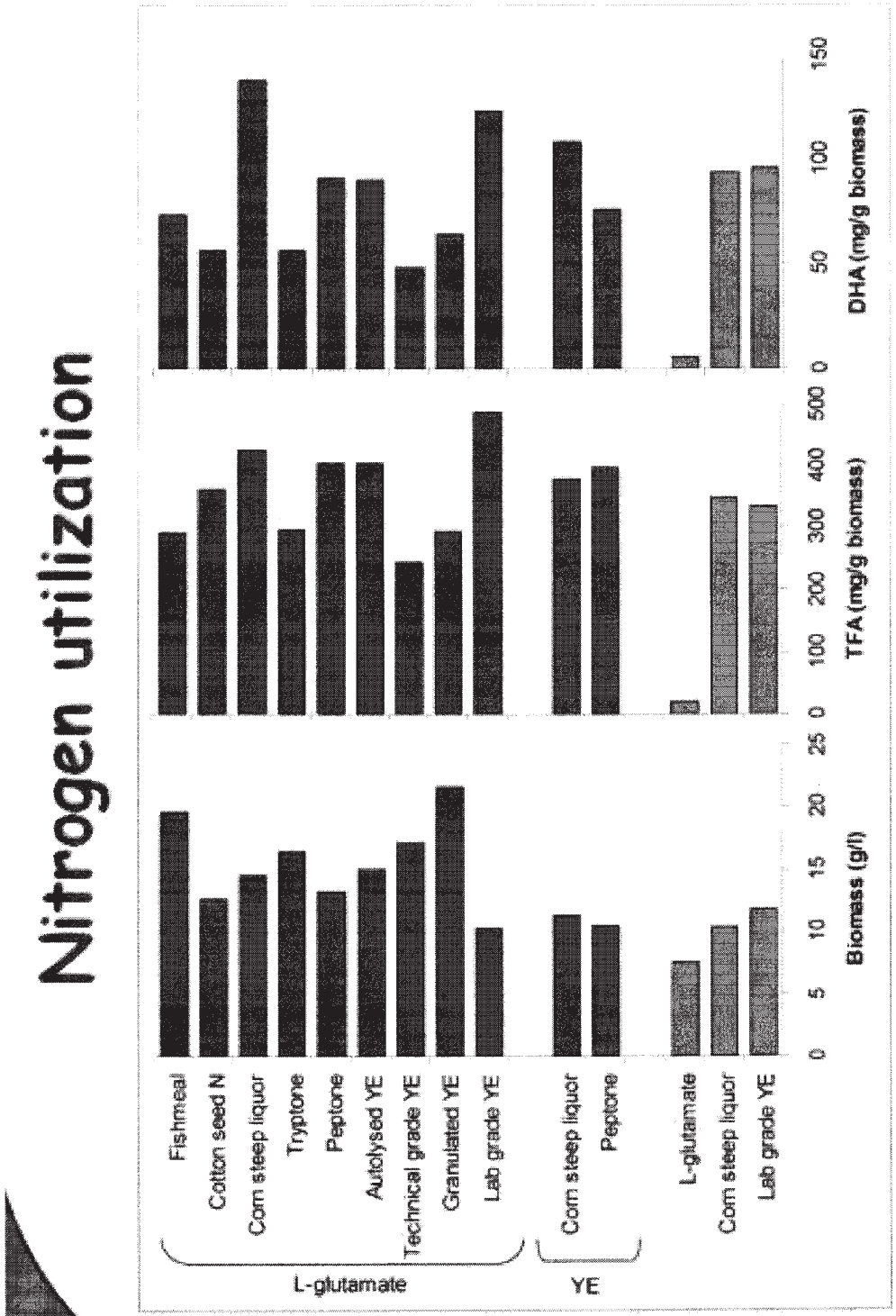
60. A pharmaceutical formulation comprising the composition of claim 58.

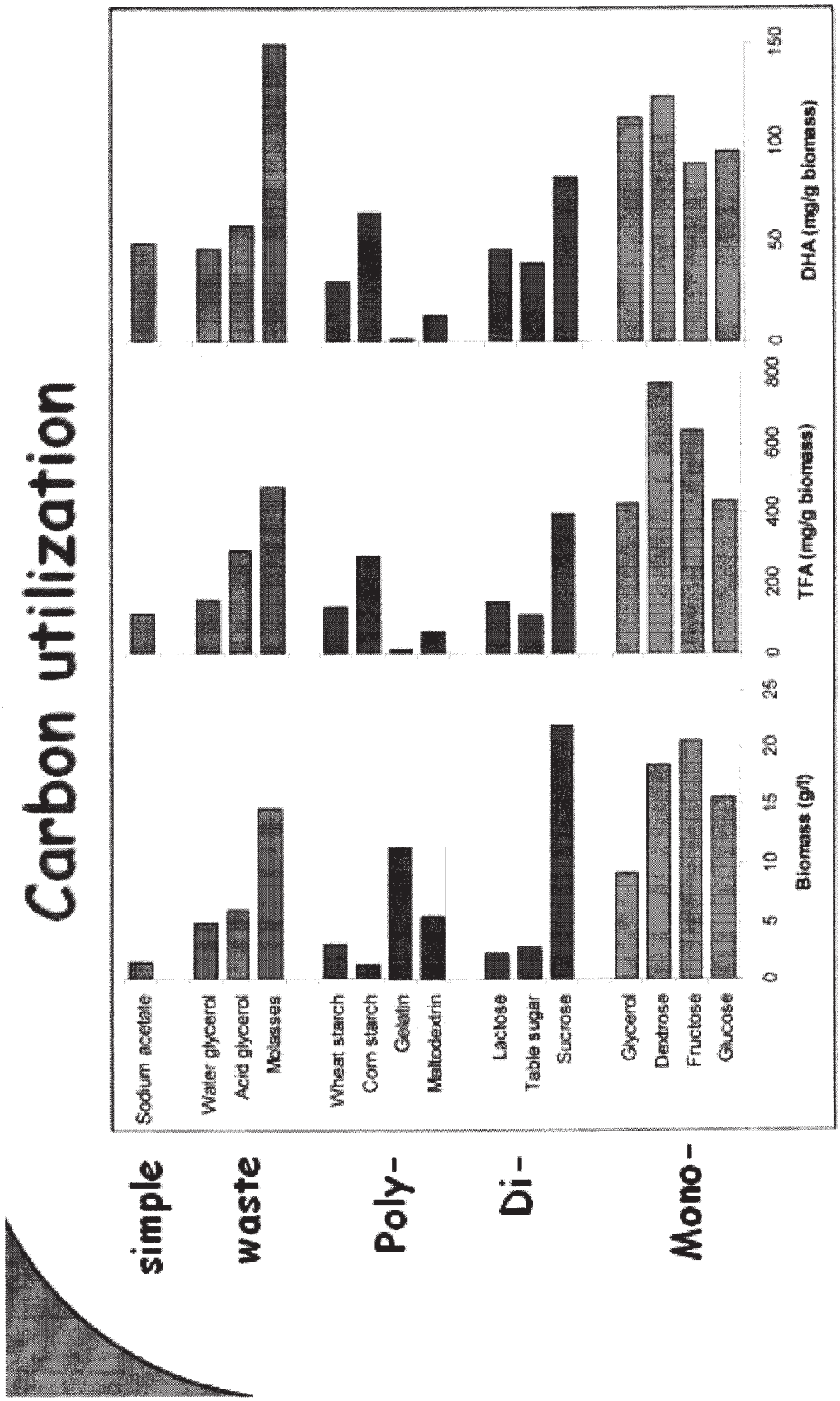

Postulated Fatty acid metabolic pathway
– fatty acid synthase
– elongase & desaturase enzymes
– polyketide synthase
fatty acid synthase
Palmitic acid (16:0)
Δ9
Palmitoleic acid (16:1)
Δ9
Stearic acid (18:0)
Δ9
Vaccenic-oleic acid (18:1)
Δ12
Linoleic acid (18:2)
Δ6
Eicosadienoic acid (20:2)
Δ5
Mead acid (20:3)
n-9 series
Δ12
Linoleic acid (18:2)
Δ6
γ-Linolenic acid (18:3)
Δ6
dihomo-γ-Linolenic acid (20:3)
Δ5
Arachidonic acid (20:4)
Δ5
Docosatetraenoic acid (22:4)
Δ4
Docosapentaenoic acid (22:5)
n-6 series
Δ15
α-Linolenic acid (18:3)
Δ6
Octadecatetraenoic acid (18:4)
Δ6
Eicosatetraenoic acid (20:4)
Δ5
Eicosapentaenoic acid (20:5)
Δ5
Docosapentaenoic acid (22:5)
Δ4
Docosahexaenoic acid (22:6)
n-3 series
ω3
desaturase
elongase

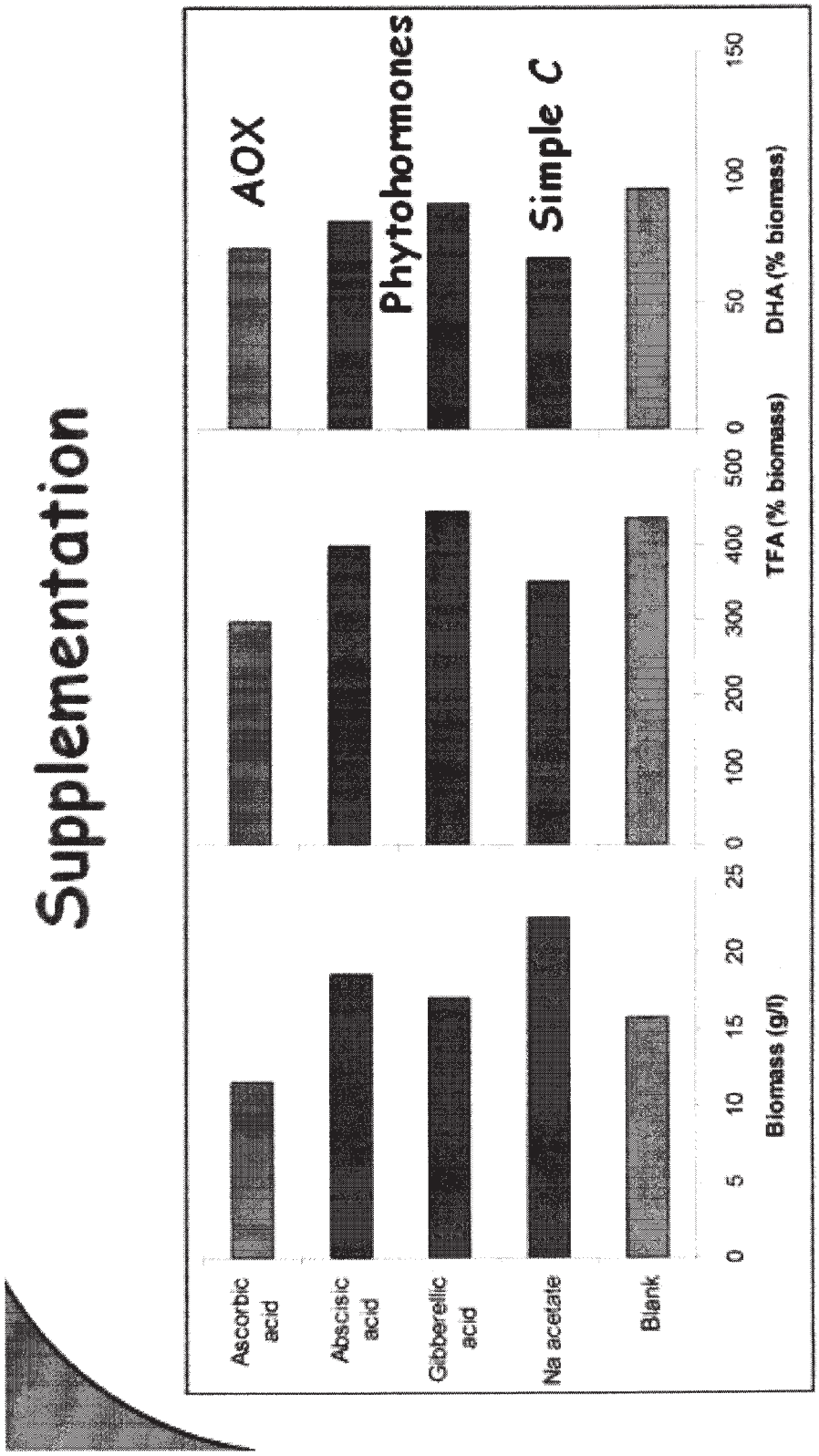

Total fatty acid:
446.7
318.2
534.6
(mg g-1 biomass)
DHA
DPA n-6
EPA
C18:1
C18:0
C16:1
C16:0
C14:0
100%
80%
60%
40%
20%
0%
Agar plate 27d
Flask 3d
Bioreactor 3d

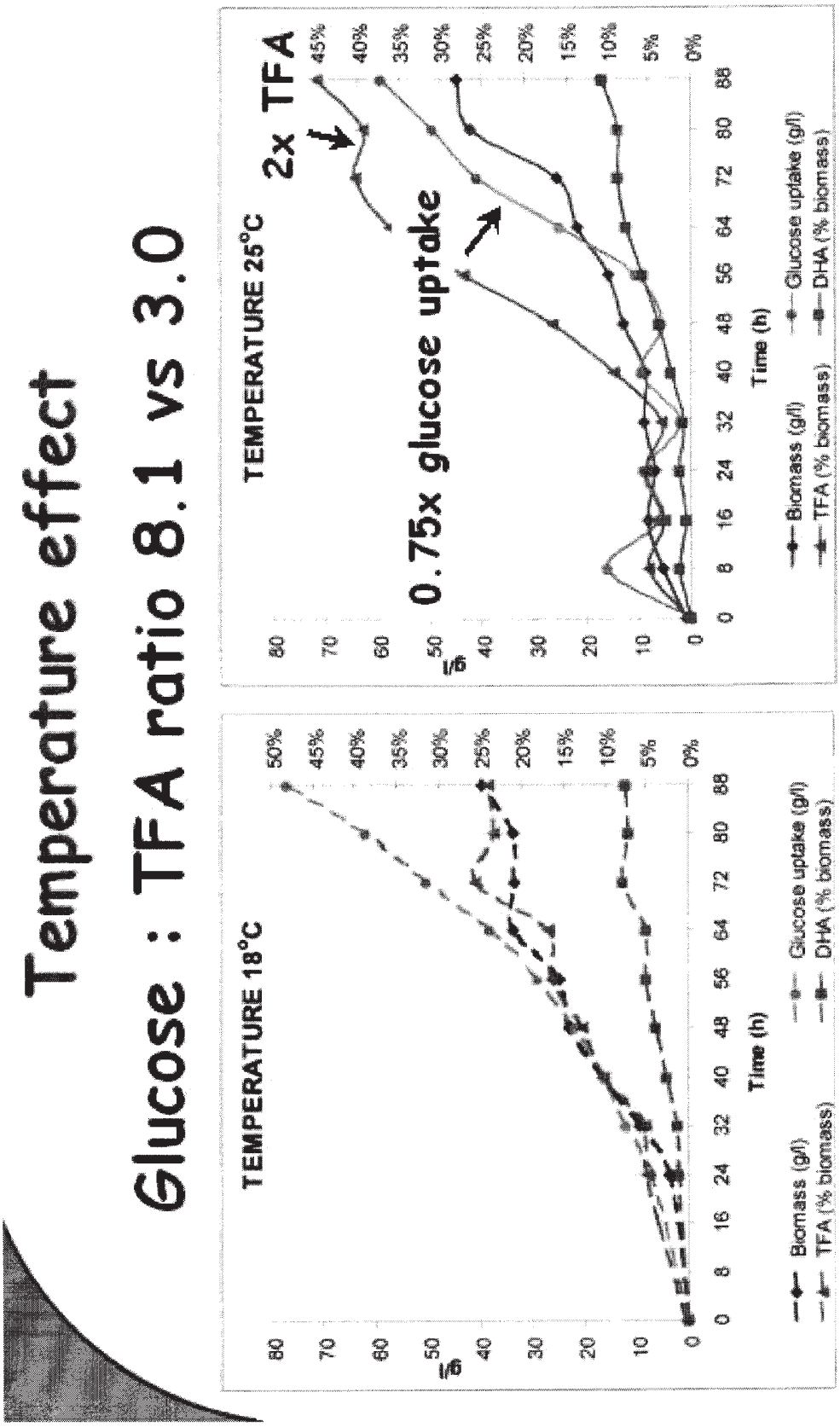

Biomass (g L-1)
TFA (% biomass)
DHA (% TFA)
Glucose (g L-1)
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
Incubation time (h)

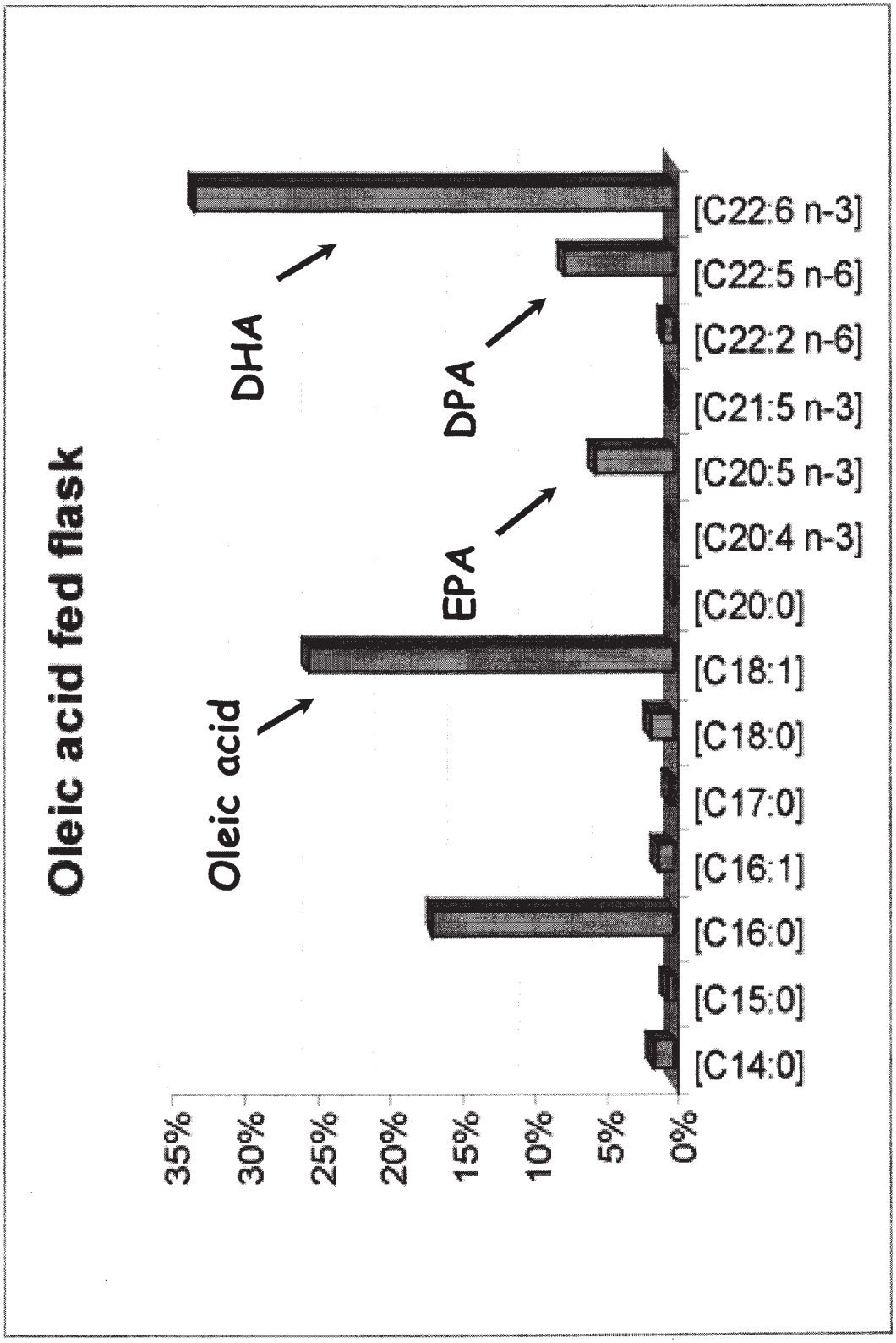

Thraustochytrium CHN-1
Thraustochytriidae MBIC 11093
Thraustochytriidae N1-27
ONCT-18
Thraustochytrium striatum T91-6
Schizochytrium KH105
Thraustochytriidae A5-20
Schizochytrium limacinum SR21
Aplanochytrium stocchinoi
Aplanochytrium kerguelense
Thraustochytrium multirudimentale
Schizochytrium minutum
Thraustochytrium pachydermum
Thraustochytriidae M4-103
Labyrinthuloides haliotidis
Thraustochytriidae N4-103
Schizochytrium aggregatum ATCC 28209
Thraustochytriidae H6-16
Thraustochytrium KK17-3
Thraustochytrium kinnei
Thraustochytriidae H1-14
Thraustochytriidae F3-1
Ulkenia profunda (#29)
Thraustochytrium aureum ATCC 34304
Ulkenia visurgensis
Japonochytrium sp. ATCC 28207
Ulkenia profunda (N3077a)
Thraustochytrium striatum ATCC 24473
0.1
969
971
999
1000
1000
1000
1000
948
1000
1000
1000
296
1000
946
1000
1000
965
282
1000
888
545
1000
1000
517


Nitrogen utilization
Fishmeal
Cotton seed N
Corn steep liquor
Tryptone
Peptone
Autolysed YE
Technical grade YE
Granulated YE
Lab grade YE
L-glutamate
Corn steep liquor
Peptone
YE
L-glutamate
Corn steep liquor
Lab grade YE
0 5 10 15 20 25
Biomass (g/l)
0 100 200 300 400 500
TFA (mg/g biomass)
0 50 100 150
DHA (mg/g biomass)


Carbon utilization
simple
waste
Poly-
Di-
Mono-
Sodium acetate
Water glycerol
Acid glycerol
Molasses
Wheat starch
Corn starch
Gelatin
Maltodextrin
Lactose
Table sugar
Sucrose
Glycerol
Dextrose
Fructose
Glucose
0 5 10 15 20 25
Biomass (g/l)
0 200 400 600 800
TFA (mg/g biomass)
0 50 100 150
DHA (mg/g biomass)


Supplementation
AOX
Phytohormones
Simple C
Ascorbic acid
Abscisic acid
Gibberellic acid
Na acetate
Blank
0 5 10 15 20 25
Biomass (g/l)
0 100 200 300 400 500
TFA (% biomass)
0 50 100 150
DHA (% biomass)


Temperature effect
Glucose : TFA ratio 8.1 vs 3.0
TEMPERATURE 18°C
TEMPERATURE 25°C
2x TFA
0.75x glucose uptake
g/l
Time (h)
Biomass (g/l)
TFA (% biomass)
Glucose uptake (g/l)
DHA (% biomass)


Oleic acid fed flask
Oleic acid
EPA
DPA
DHA
[C22:6 n-3]
[C22:5 n-6]
[C22:2 n-6]
[C21:5 n-3]
[C20:5 n-3]
[C20:4 n-3]
[C20:0]
[C18:1]
[C18:0]
[C17:0]
[C16:1]
[C16:0]
[C15:0]
[C14:0]
35%
30%
25%
20%
15%
10%
5%
0%

Media	Lipid (mg/g)	DHA (% Lipid)	EPA (% Lipid)	DPAn6 (% Lipid)	EPA/DPAn6 ratio	EPA/DHA ratio
Control	344	31	2.8	10.1	0.278	0.090
0.2 g/L Toluic Acid	450	31	6.6	5.9	1.119	0.213

Antagonist	[C14:0]	% TFA	[C14:1]	% TFA	[C15:0]	% TFA	[C15:1]	% TFA	[C16:0]	% TFA	[C16:1]	% TFA	[C17:0]	% TFA	[C18:0]	% TFA
Control	36.91	11.27	0.00	0.23	20.36	3.92	1.19	0.43	209.79	45.57	12.93	11.63	5.79	1.16	8.76	1.84
Sethoxydim (100 uM)	58.53	10.97	0.15	0.03	29.34	5.50	2.80	0.53	216.18	40.52	48.97	9.18	7.93	1.49	7.73	1.45
Control	59.46	11.10	0.00	0.00	20.56	3.84	0.57	0.11	270.63	50.52	10.35	1.93	4.57	0.85	8.30	1.55
Cerulenin (20 mg/l)	65.33	11.03	0.00	0.00	19.16	3.23	0.62	0.10	290.18	48.98	13.02	2.20	4.40	0.74	11.00	1.86
Oleic acid (2 g/l)	2.11	1.38	0.00	0.00	0.74	0.49	0.00	0.00	25.35	16.67	1.61	1.06	0.43	0.28	2.37	1.56
Oleic acid (2 g/l) Glucose (5 g/l)	16.96	8.40	0.00	0.00	1.48	0.73	0.00	0.00	67.00	33.18	8.52	4.22	0.34	0.17	3.22	1.60
Control	90.55	24.46	0.10	0.03	9.00	2.43	0.00	0.00	158.50	42.82	1.71	0.46	1.13	0.31	4.67	1.26
Capsaicin (0.1 g/L)	79.08	21.86	1.01	0.28	3.31	0.91	0.83	0.23	87.52	24.19	65.04	17.98	0.70	0.19	5.78	1.60
Control	53.08	9.70	0.00	0.00	53.80	9.83	0.61	0.11	237.91	43.48	8.33	1.52	12.53	2.29	8.73	1.59
Toluic acid (0.2 g/L)	22.41	11.19	0.00	0.00	29.90	14.93	0.55	0.27	53.86	26.90	4.72	2.36	5.38	2.69	3.19	1.59
Control	32.44	8.95	0.00	0.00	35.50	9.79	0.46	0.13	118.97	32.80	7.05	1.94	6.86	1.89	4.63	1.28
Norflurazon (0.2 g/L)	53.14	11.08	0.59	0.12	15.18	3.16	1.60	0.33	194.80	40.61	51.10	10.65	4.93	1.03	7.30	1.52
Control	62.90	12.36	0.63	0.12	13.81	2.71	1.76	0.35	220.04	43.24	79.08	15.54	4.05	0.80	8.17	1.61

Antagonist	[C18:1w9]	% TFA	[C18:1w7]	% TFA	[C20:0]	% TFA	[C20:1]	% TFA	[C20:2w6]	% TFA	[C20:3w6]	% TFA	[C20:4w6]	% TFA	[C20:3w3]	% TFA
Control	0.00	0.00	12.98	2.89	1.72	0.36	0.91	0.12	1.64	0.38	1.27	0.14	1.24	0.13	0.00	0.00
Sethoxydim (100 uM)	0.96	0.18	13.21	2.48	1.09	0.20	0.00	0.00	0.75	0.14	0.51	0.10	1.09	0.20	0.00	0.00
Control	0.71	0.13	4.99	0.93	2.03	0.38	0.00	0.00	0.90	0.17	0.82	0.15	0.88	0.16	0.00	0.00
Cerulenin (20 mg/l)	1.71	0.29	7.35	1.24	2.40	0.41	1.15	0.19	1.00	0.17	1.21	0.20	1.79	0.30	0.00	0.00
Oleic acid (2 g/l)	38.55	25.36	4.08	2.68	0.00	0.00	0.00	0.00	0.77	0.51	0.92	0.61	1.09	0.72	0.00	0.00
Oleic acid (2 g/l) Glucose (5 g/l)	19.17	9.49	8.98	4.45	0.68	0.34	0.60	0.30	0.72	0.36	0.60	0.30	0.93	0.46	0.00	0.00
Control	0.55	0.15	0.95	0.26	1.33	0.36	0.00	0.00	0.23	0.06	0.47	0.13	0.81	0.22	0.00	0.00
Capsaicin (0.1 g/L)	0.00	0.00	24.13	6.67	0.73	0.20	0.00	0.00	0.84	0.23	0.81	0.22	2.83	0.78	0.00	0.00
Control	0.54	0.10	6.95	1.27	1.45	0.26	0.00	0.00	0.19	0.03	1.35	0.25	0.96	0.17	0.00	0.00
Toluic acid (0.2 g/L)	0.23	0.11	7.37	3.68	0.46	0.23	0.29	0.14	1.58	0.79	0.34	0.17	1.81	0.90	0.16	0.08
Control	0.36	0.10	5.95	1.64	0.80	0.22	0.05	0.01	0.73	0.20	0.43	0.12	1.37	0.38	0.11	0.03
Norflurazon (0.2 g/L)	0.00	0.00	16.96	3.53	0.78	0.16	0.08	0.02	0.61	0.13	0.87	0.18	0.90	0.19	0.00	0.00
Control	0.22	0.04	16.38	3.22	1.17	0.23	0.00	0.00	0.77	0.15	0.55	0.11	0.74	0.14	0.00	0.00

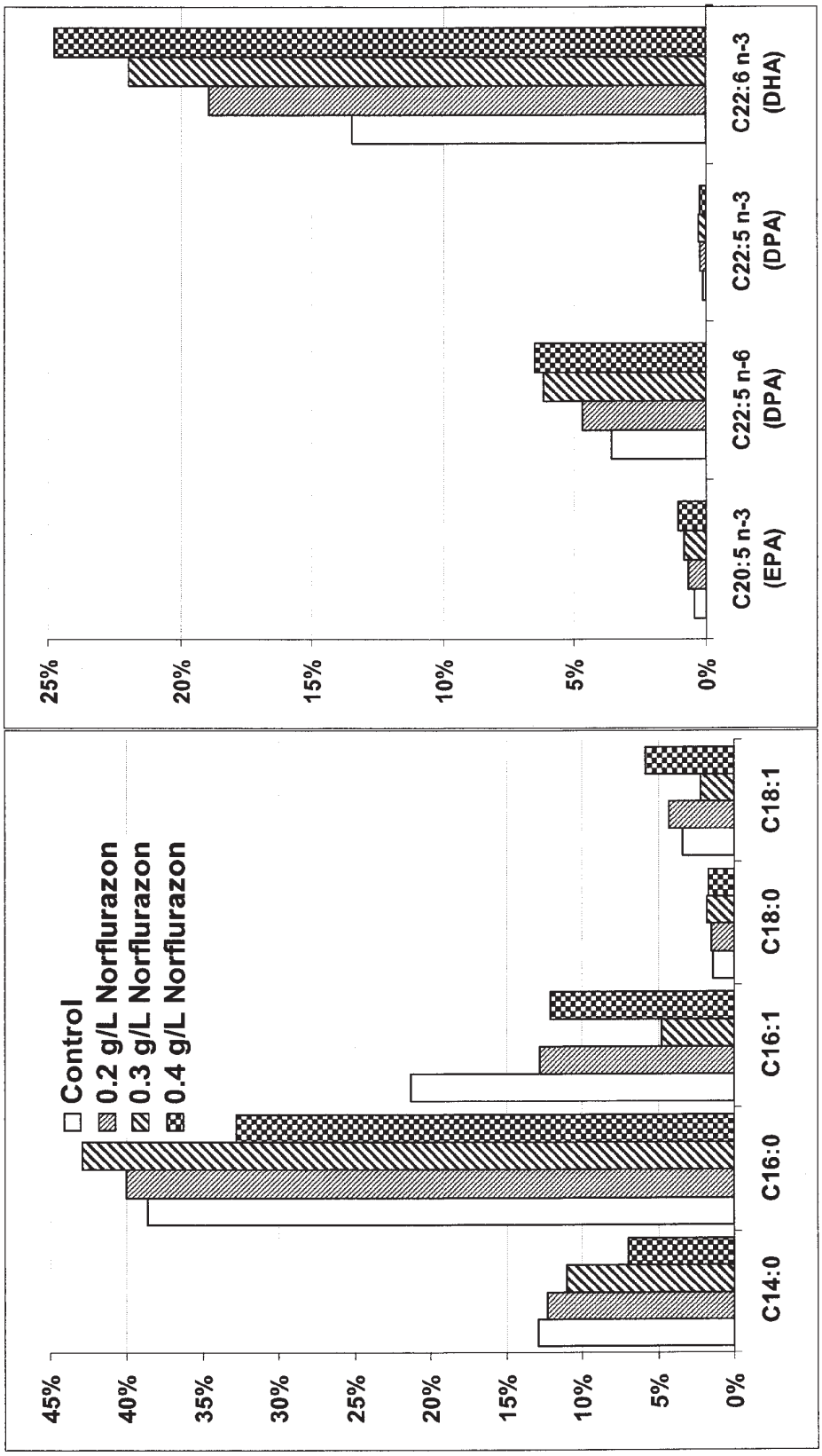
Antagonist	[C20:4w3]	% TFA	[C20:5w3]	% TFA	[C22:0]	% TFA	[C21:5w3]	% TFA	[C22:2w6]	% TFA	[C22:4w6]	% TFA	[C22:5w6]	% TFA	[C22:5w3]	% TFA
Control	3.46	0.26	1.09	0.38	0.00	0.00	4.47	1.12	1.25	0.20	1.21	0.16	45.95	3.88	4.34	0.35
Sethoxydim (100 uM)	1.97	0.37	4.15	0.78	0.00	0.00	5.84	1.09	0.00	0.00	0.00	0.00	28.04	5.26	1.25	0.24
Control	1.84	0.34	3.85	0.72	0.52	0.10	6.78	1.27	0.71	0.13	0.00	0.00	29.29	5.47	0.84	0.16
Cerulenin (20 mg/l)	2.11	0.36	6.94	1.17	0.64	0.11	7.32	1.24	0.84	0.14	0.00	0.00	34.53	5.83	1.39	0.23
Oleic acid (2 g/l)	0.00	0.00	8.32	5.47	0.00	0.00	0.31	0.20	1.03	0.68	0.92	0.60	11.52	7.58	1.50	0.98
Oleic acid (2 g/l) Glucose (5 g/l)	0.49	0.24	5.25	2.60	0.00	0.00	1.60	0.79	0.87	0.43	0.57	0.28	13.05	6.46	0.78	0.39
Control	1.02	0.27	2.30	0.62	0.38	0.10	3.74	1.01	0.53	0.14	0.54	0.14	21.65	5.85	0.52	0.14
Capsaicin (0.1 g/L)	0.98	0.27	5.01	1.38	0.00	0.00	0.00	0.00	0.60	0.17	1.66	0.46	18.78	5.19	1.12	0.31
Control	1.62	0.30	2.65	0.48	0.45	0.08	0.00	0.00	0.70	0.13	0.39	0.07	35.76	6.53	1.21	0.22
Toluic acid (0.2 g/L)	0.72	0.36	5.91	2.95	0.00	0.00	0.78	0.39	0.11	0.06	0.93	0.46	11.92	5.95	1.09	0.54
Control	1.39	0.38	4.21	1.16	0.00	0.00	1.57	0.43	0.33	0.09	0.67	0.19	34.21	9.43	0.98	0.27
Norflurazon (0.2 g/L)	1.17	0.24	3.65	0.76	0.25	0.05	0.00	0.00	0.15	0.03	0.81	0.17	25.09	5.23	0.90	0.19
Control	0.68	0.13	2.65	0.52	0.37	0.07	0.00	0.00	0.54	0.11	1.16	0.23	20.25	3.98	0.69	0.13

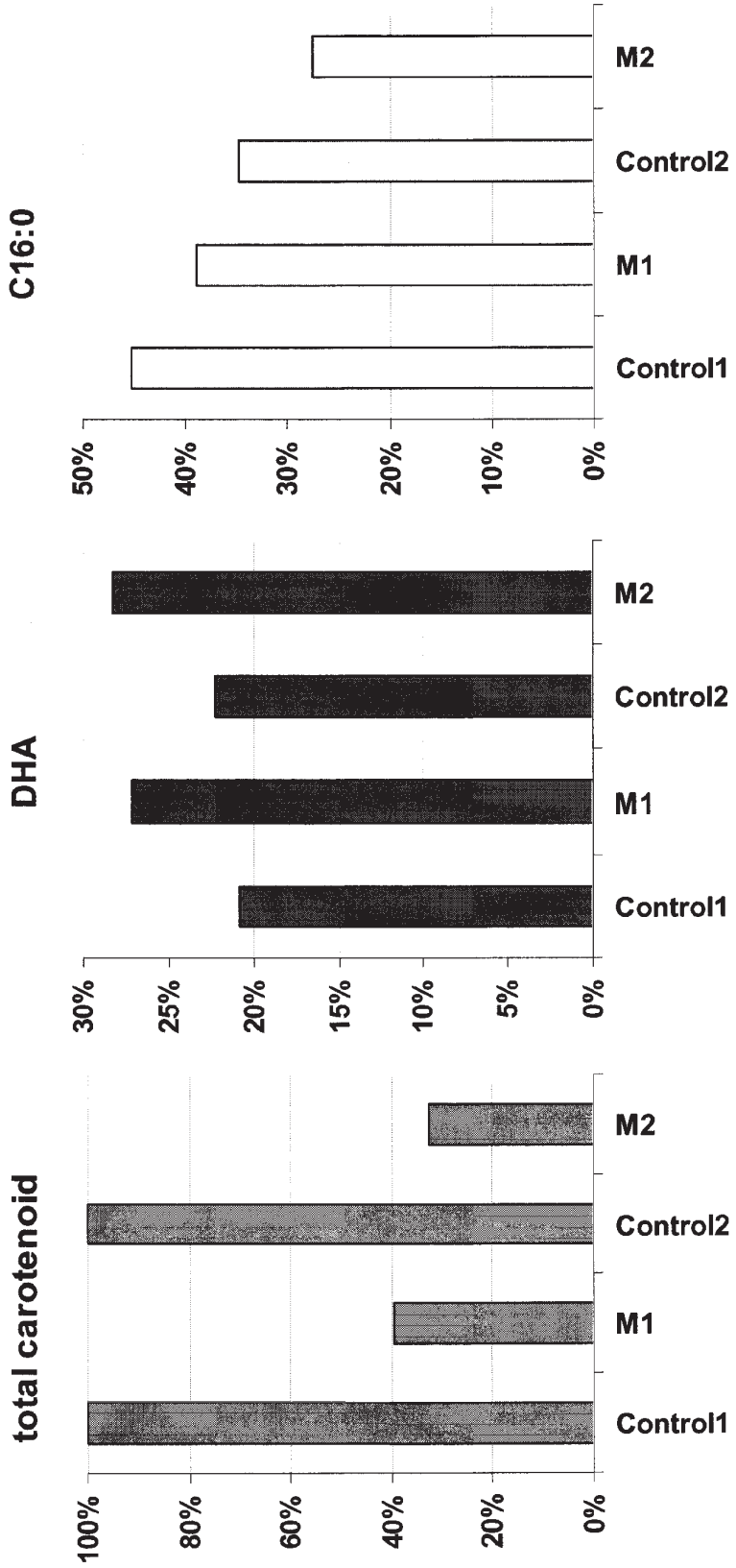
Antagonist	[C24:0]	% TFA	[C22:6w3]	% TFA	[C24:1w9]	% TFA	[C23:0]	% TFA
Control	0.00	0.00	149.96	13.54	0.00	0.00	1.98	0.27
Sethoxydim (100 uM)	0.00	0.00	103.00	19.31	0.00	0.00	1.06	0.20
Control	0.00	0.00	107.04	19.98	0.00	0.00	0.88	0.16
Cerulenin (20 mg/l)	0.00	0.00	118.32	19.97	0.00	0.00	1.34	0.23
Oleic acid (2 g/l)	0.00	0.00	50.42	33.16	0.00	0.00	0.00	0.00
Oleic acid (2 g/l) Glucose (5 g/l)	0.00	0.00	49.78	24.65	0.00	0.00	0.00	0.00
Control	0.00	0.00	69.48	18.77	0.00	0.00	0.00	0.00
Capsaicin (0.1 g/L)	0.00	0.00	60.99	16.86	0.00	0.00	0.00	0.00
Control	0.48	0.09	117.55	21.48	0.00	0.00	0.00	0.00
Toluic acid (0.2 g/L)	0.07	0.03	46.12	23.03	0.36	0.18	0.00	0.00
Control	0.26	0.07	103.28	28.48	0.11	0.03	0.00	0.00
Norflurazon (0.2 g/L)	0.00	0.00	98.85	20.61	0.00	0.00	0.00	0.00
Control	0.44	0.09	71.90	14.13	0.00	0.00	0.00	0.00

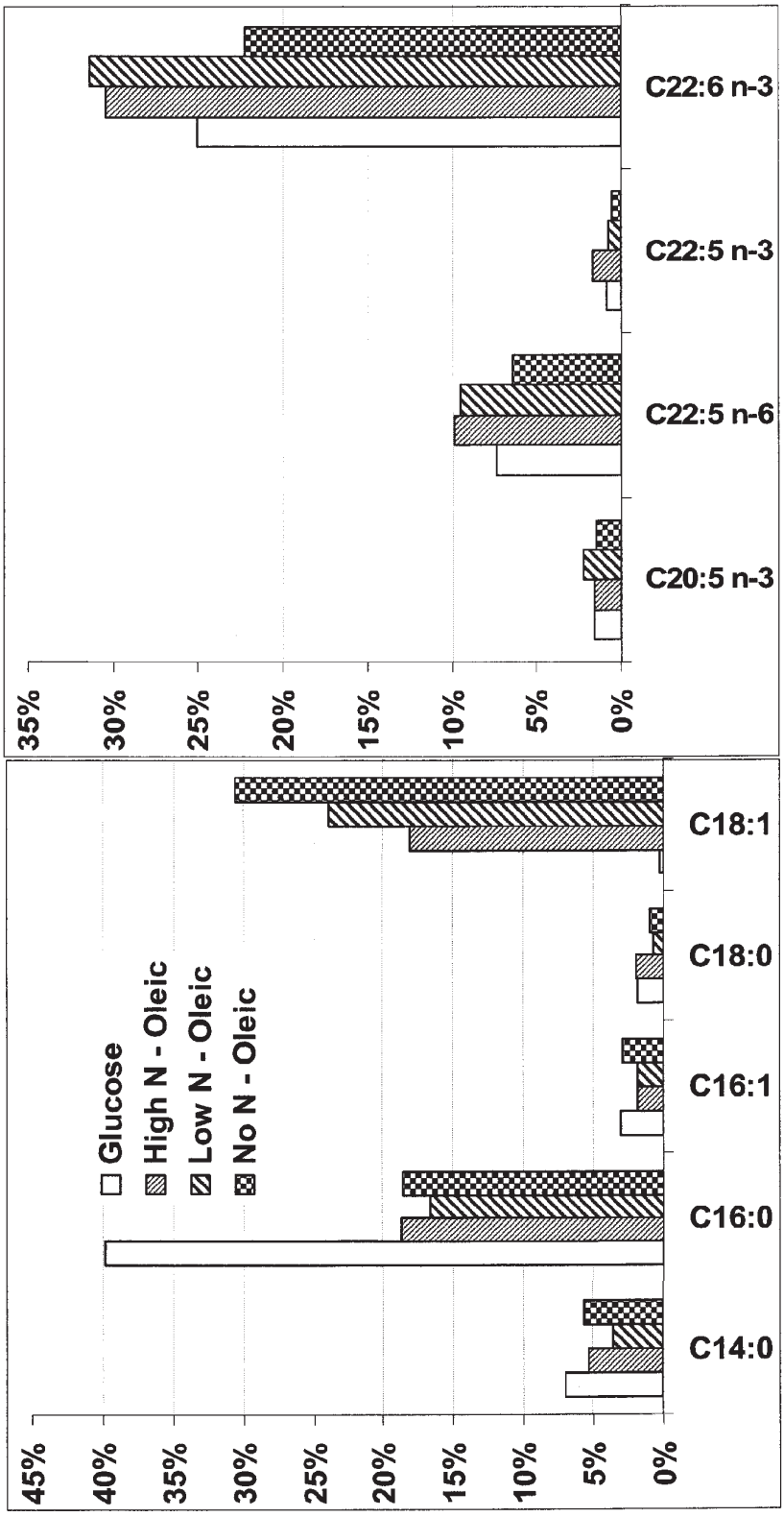
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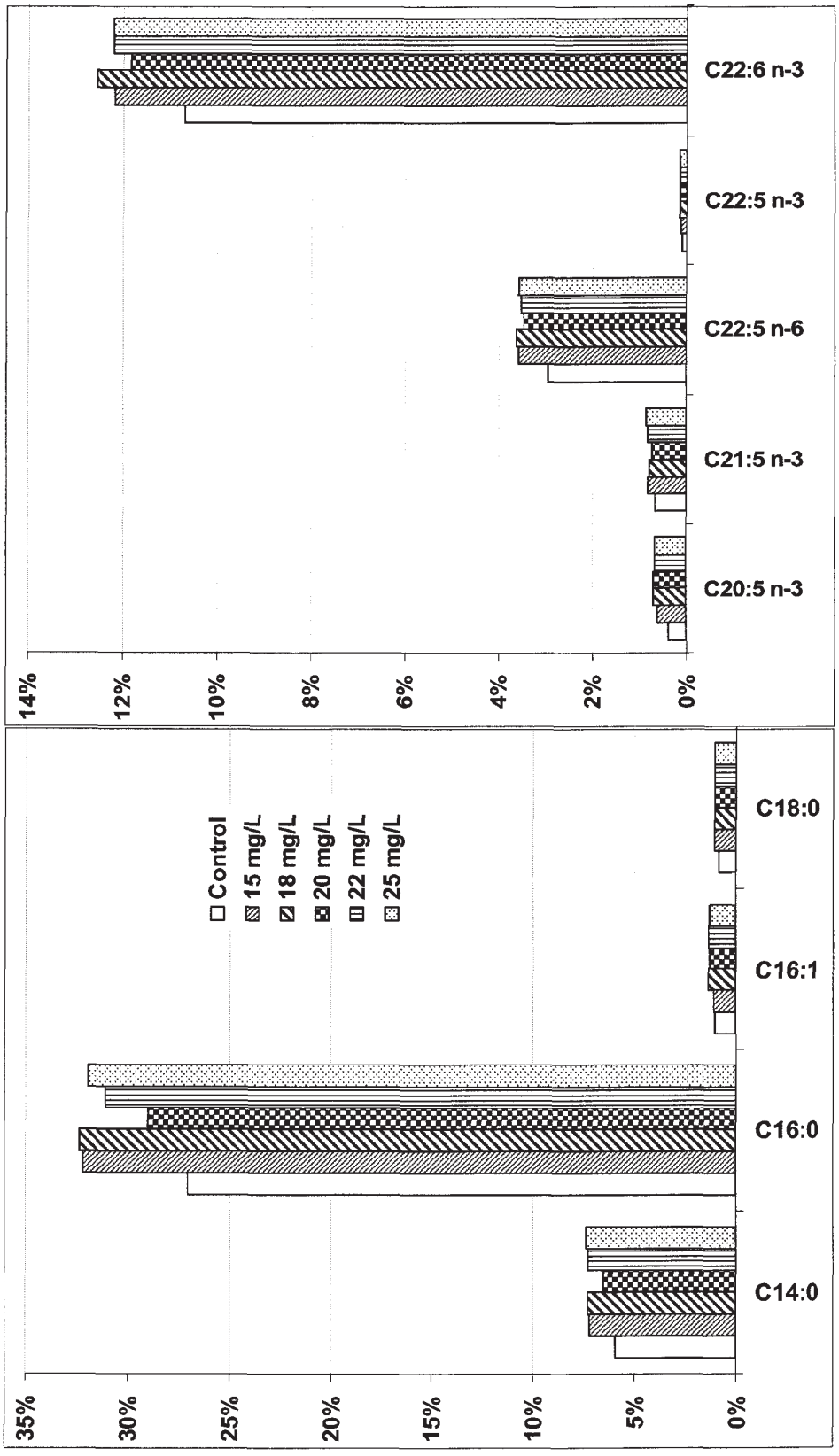
[Cx:x] denotes the mg/g amount of each fatty acid identified via GC FAMEs analysis

% TFA denotes the % of the total fatty acid content for each fatty acid represented


Control
0.2 g/L Norflurazon
0.3 g/L Norflurazon
0.4 g/L Norflurazon
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
C14:0
C16:0
C16:1
C18:0
C18:1
25%
20%
15%
10%
5%
0%
C20:5 n-3 (EPA)
C22:5 n-6 (DPA)
C22:5 n-3 (DPA)
C22:6 n-3 (DHA)


C16:0
M2
Control2
M1
Control1
50%
40%
30%
20%
10%
0%
DHA
M2
Control2
M1
Control1
30%
25%
20%
15%
10%
5%
0%
total carotenoid
M2
Control2
M1
Control1
100%
80%
60%
40%
20%
0%


C22:6 n-3
C22:5 n-3
C22:5 n-6
C20:5 n-3
35%
30%
25%
20%
15%
10%
5%
0%
C18:1
C18:0
C16:1
C16:0
C14:0
Glucose
High N - Oleic
Low N - Oleic
No N - Oleic
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%


C22:6 n-3
C22:5 n-3
C22:5 n-6
C21:5 n-3
C20:5 n-3
14%
12%
10%
8%
6%
4%
2%
0%
Control
15 mg/L
18 mg/L
20 mg/L
22 mg/L
25 mg/L
C18:0
C16:1
C16:0
C14:0
35%
30%
25%
20%
15%
10%
5%
0%